SOME CHEMICAL PROPERTIES OF GLYCINE ACCEPTOR RNA FROM RAT LIVER

BY

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To
My Mother

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ABSTRACT

Rat liver sRNA has been fractionated by chromatography on amino-ethyl (AE-50) cellulose column. Several eluting systems have been used in the chromatography, including the buffer system of Zubay, who reported a 70% purification of leucine acceptor RNA by chromatography on amino-ethyl cellulose column. Elution of glycyl RNA from the column was favoured by solutions of more alkaline pH (7·0-9·0). A 2·5-fold purification of glycyl RNA has been obtained with a pH gradient from pH 6·0-8·5 in 0·5 M potassium phosphate buffer. This corresponded to a purification with respect to the glycine acceptor activity of only 6·2%. This method for purification of glycyl RNA appeared to be poor for a number of reasons; 1) instability of the glycine-sRNA ester, 2) incomplete Schiff base complex formation between oxidized sRNA and the amino-ethyl cellulose, and 3) the β-elimination reaction which labilized the Schiff base complex.

Stabilization of the glycyl RNA ester by acetylation has been demonstrated. The conditions of acetylation do not destroy the glycine acceptor activity. The acetylated glycyl RNA had a half-life (pH 7·5, 37°C) about ten times that of unacetylated glycyl RNA.

The enzyme-catelyzed reconstitution of the glycine acceptor activity of pancreatic RNase-treated and partially pyrophosphorolyzed sRNAs have been studied. The sRNA which had been treated briefly with a small amount of pancreatic RNase (0·0001 µg/ml) was reconstituted in the presence of all four nucleoside triphosphates and "pH 5 enzymes" fraction. This suggests that the amino acid

acceptor end of glycyl RNA is particularly sensitive to digestion by RNase. In the case of partially pyrophosphorolyzed sRNA, only CTP and ATP were required to reconstitute glycine acceptor activity. This is in agreement with the findings of other investigators with other acceptor RNAs and suggests that the terminal nucleotide sequence for glycyl RNAmay also be pCpCpA.

Direct evidence has been obtained by the finger-print technique for the existence of the acetyl glycine ester of adenosine and the sequence pCpA-acetylglycine in a pancreatic RNase digest of acetylated C^{14}-glycine-labelled sRNA. It may be concluded that the terminal sequence of nucleotides at the amino acid acceptor end of glycyl RNA is pPypCpA, where Py represents a pyrimidine. This is in agreement with the findings of other investigators with other specific amino acyl RNAs.

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INTRODUCTION

Role of sRNA in protein biosynthesis

It was first discovered by Hoagland (1) in 1957 that activated amino acids became bound to the soluble ribonucleic acid fraction derived from rat liver homogenate before they were incorporated into ribosomal proteins. The amino acyl RNA compound functions as a true intermediate because it transfers activated labelled amino acids into ribosomal protein without dilution by unlabelled amino acids (2). This soluble RNA fraction has been isolated from yeast (3), E. coli (4, 5) and mammalian livers (6, 7).

At present the most widely accepted hypothesis for protein biosynthesis is that it appears to occur in three steps (8), namely: a) activation of the carboxyl groups of amino acids with ATP by specific activating enzymes in the presence of Mg^{++} ions to form enzyme-bound amino acyl adenylates,

$$*En + ATP + aa \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} En\text{-}AMP\text{-}aa + PP$$

b) acceptance of the amino acyl moieties of the enzyme-bound amino acyl adenylates by specific sRNA's (acceptor RNAs) to form amino acyl RNA esters,

$$En\text{-}AMP\text{-}aa + sRNA \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} En + AMP + sRNA\text{-}aa$$

c) transfer of amino acids from amino acyl RNAs in the presence of GTP into peptide linkages in the ribosomal particles. This step is catalyzed by at least two enzymes, called transfer enzymes. One enzyme has GTPase activity. The other enzyme probably catalyzes peptide bond formation.

*En is used to refer to enzyme, in this case, amino acyl RNA synthetase.

$$\text{sRNA-aa} + \text{ribosomes} \xrightarrow{\text{GTP}} \text{sRNA} + \text{ribosomal-protein}$$

According to the adaptor hypothesis (9) the sRNA acts as an adaptor conveying the amino acid to the appropriate site on the ribosomal messenger RNA. Messenger RNA, carrying the genetic information from DNA, attaches to the ribosomes and is believed to provide the site or template for protein biosynthesis. The interaction between the amino acyl RNA and the messenger RNA probably occurs by hydrogen bonding between complementary base sequences in the two species of RNA. There is both genetic and chemical evidence to support the hypothesis that a trinucleotide sequence "codon" in messenger RNA codes for one amino acid. Accordingly, it would be expected that a complementary triplet base sequence in a specific acceptor RNA molecule would interact with the codon. When two amino acyl RNA molecules are aligned on the template peptide bond formation can take place. There is evidence that this occurs first in the region of the messenger corresponding to the N-terminal amino acid sequence in the polypeptide coded for, and that the wave of peptide bond formation proceeds to the C-terminal region as the appropriate amino acyl RNA molecules are aligned on the template (119).

A great deal of evidence supports the above mechanism. Hoagland (10) in 1958 with a particle free extract of rat liver demonstrated that radioactive inorganic pyrophosphate (^{32}PP) would exchange with ATP in the presence of Mg^{++}, amino acid, ATP and rat liver extract. This ATP-^{32}PP exchange is thus amino acid-dependent. The exchange is furthermore additive with respect to

the different amino acids. This amino acid-dependent ATP-^{32}PP exchange reaction has also been demonstrated with extracts from many mammalian tissues as well as microbial sources (11-13).

The isolation of tryptophan adenylate (14) from an incubation mixture containing the purified tryptophan activating enzyme, tryptophan, ATP and Mg^{++} ions has been reported. This strongly supports Hoagland's hypothesis that an intermediate enzyme-bound adenylate is formed during the activation step in protein biosynthesis. Furthermore, amino acyl adenylates have been prepared synthetically (15) and have been shown to take part in the reversal activation reaction (step a) and to take part in the formation of amino acyl RNA ester (step b).

Some of the enzymes responsible for the activation of amino acids have been isolated from plant, microbial and animal sources (16-20). The isolation of a highly purified tryptophanyl RNA synthetase (20), seryl RNA synthetase (22) and glycyl RNA synthetase (23) indicates further that the amino acyl RNA synthetase is specific for the activation of a particular amino acid. The same enzyme that activates the amino acid and catalyzes the amino acid-dependent ATP-^{32}PP exchange also catalyzes the transfer of the amino acyl moiety from the enzyme-bound amino acyl adenylate to a specific amino acid acceptor RNA (24-26). These enzymes are thus designated amino acid activating enzymes or amino acyl RNA synthetases. The latter term is considered to be a better one because there are other amino acid activating enzymes, for instance, the activation of the γcarboxyl group of glutamic acid (27), not involved in protein biosynthesis.

Recently Berg (28) and Lagerkvist et al (29) have shown that there is species specificity in the interaction between an amino acyl RNA synthetase and sRNA. The amino acyl RNA synthetase from rat liver and yeast will esterify leucine to sRNA from rat liver or yeast but not to E. coli sRNA (30). This implies that the sRNA and the amino acyl synthetase from rat liver and yeast probably differ in structure from those of E. coli preperations.

Each amino acid is linked enzymatically to a specific sRNA chain through an ester bond between the carboxyl group of the amino acid and the 3' hydroxyl group (31) of the ribosyl moiety of a terminal AMP residue of sRNA (31-34). The identification of the amino acyl ester linkage with the terminal nucleotide of sRNA is supported by the fact that oxidation of the cis-hydroxyl group of the terminal ribose moiety with periodate destroys the acceptor activity for all amino acids (35). The isolation of leucine adenosine ester from a pancreatic RNase digest of leucyl RNA strongly supports the conclusion that the amino acid is bound to the terminal AMP residue of sRNA (34, 36).

Chapeville et al (37) have converted by reduction cysteinyl RNA, prepared enzymically, to alanyl RNA without breaking the ester linkage. When this alanyl RNA-hybrid was incubated with a ribosomal amino acid incorporating system the alanine was incorporated into protein in the position where cysteine was normally incorporated. This experiment was carried out with the rabbit reticulocyte system which makes mainly hemoglobin. Thus it is the sRNA but not the amino acid, which participates in the coding, the sRNA adapting the attached amino acid to the template.

When synthetic polyuridylic acid was added to a cell free amino acid incorporating system it was found that a 1,000 fold stimulation of L-phenylalanine incorporation occurred (38). This was interpreted to mean that the synthetic poly U can perform the role of messenger RNA. Poly U must contain the code words for phenylalanine. It is believed (39) that a unique sequence of three nucleotides in the messenger RNA is required to code one amino acid; in this case the code words for phenylalanine would be UUU. By the similar experiment using synthetic polynucleotides with different base compositions the code words for all other natural occurring amino acids have been worked out. Very recently, Nirenberg and Leder (40) have shown that, in the presence of specific trinucleotides, only the corresponding specific amino acyl RNA molecules bind to the ribosomes, in the absence of peptide bond formation. With the three trinucleotides corresponding to the code word for valine (2U,G), namely, GUU, UGU and UUG, only in the presence of the first of these did valyl RNA become bound to the ribosomal particles. This suggests strongly that the code word for valine is GUU. Thus, a method now seems to be available for not only redetermining all of the code words by an entirely new method, but also for determining the sequences of the code words for each amino acid. Furthermore, the finding that a triplet sequence is sufficient for this amino acyl RNA binding to the ribosomes also provides chemical evidence in support of the hypothesis of a triplet genetic code.

Evidence for separate acceptor RNAs for each amino acid

Unfractionated sRNAs isolated from a variety of sources have been shown to have all of amino acid acceptor activities (41). There is a definite upper limit of incorporation for each amino acid and the C^{14}-amino acids are incorporated into sRNA independently of each other, that is to say, the incorporation of amino acids into sRNA is additive (42). Furthermore, it has been shown, for example, that addition of non-radioactive threonine does not inhibit incorporation of radioactive tyrosine into sRNA and vice versa. Since there is only one binding site in the sRNA molecule it is apparent that each amino acid must be associated with and incorporated into a specific sRNA chain.

Recent work on the fractionation of crude sRNA preparations has, in fact, yielded several fractions enriched with respect to particular amino acid acceptor activities. Holley et al (43), using counter current distribution technique, have obtained relatively pure alanine-, valine- and tyrosine-specific sRNAs from yeast. Valyl RNA from yeast has been fractionated by a combined chromatographic and chemical procedure (44). Leucyl RNA from E. coli has been obtained by chromatography on an amino-ethyl cellulose column (45), and seryl RNA from yeast has been separated by partition chromatography on a Sephadex column (21). Such results of fractionation of sRNA show that separate amino acid acceptor RNAs exist for each amino acid.

When sRNA was fractionated by counter current distribution at least four types of sRNA were found to accept L-leucine (46) and several other amino acids were accepted by more than one type of

sRNA. Further evidence indicates that two of the leucyl RNAs have different base compositions and are coded by different triplets. If codification for a particular amino acid is not confined uniquely to a triplet, the code is said to be degenerate (47).

Physical properties of sRNA

Ultracentrifugal studies from several laboratories (5, 48, 49) revealed that unfractionated sRNA is relatively homogeneous with respect to molecular size. The sedimentation constant has been found to be 4 S and the weight average molecular weight about 25,000 ± 2,000 corresponding to about 70 nucleotides per molecule (45, 51). In fact, Luborsky and Centoni (48) found that at low concentration, sRNA preparation (rabbit liver) showed about 12% sedimentation heterogeneity. This small physical heterogeneity may reflect differences in shape as well as in size among the various sRNA chains.

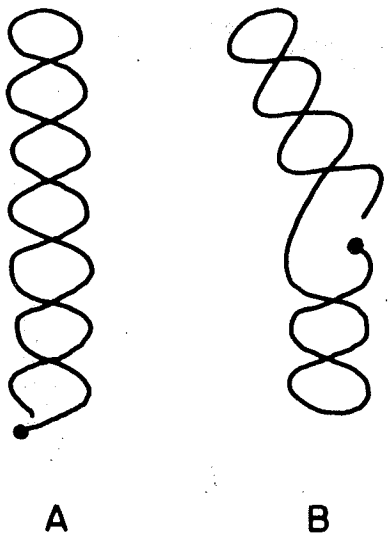
The X-ray diffraction patterns from sRNA obtained by Rich and Watson (52) suggested that sRNA molecules have identical and highly regular structure. Spencer (53) also suggested from the X-ray diffraction studies of crystallized yeast sRNA, that the molecules possess a very regular helical structure. The crystallized material, however, did not have the commonly reported sedimentation constant of 4 S and molecular weight of 25,000, but rather it has a sedimentation constant of about 2·5 S and an average molecular weight of about 10,000. It was subsequently found that sRNA preparations which have the 4 S sedimentation constant do not crystallise (53). Crystallization was induced when 4 S material was heated to 100°C in dilute solution. Under these conditions

the sedimentation constant falls from 4 S to about 2·5 S and the X-ray diffraction data suggest that crystalline 2·5 S material has mainly a double helical structure. No conclusion can be drawn about the secondary structure of native sRNA from these X-ray diffraction data.

When sRNA is heated in dilute salt solution it exhibits marked hyperchromicity indicating a considerable splitting of base-pairs in the molecule. The profile obtained by plotting the increase in optical density against temperature is often referred to as the melting curve. The degree of hyperchromicity reveals the proportion of bases existing in the double helical configuration (54). Several estimations of hyperchromicity of sRNA in solution have been reported (49, 55) indicating that approximately 45-75% of the material is helical. On digestion of only 40-50% of the sRNA chain by snake venom phosphodiesterase which is known to cleave sRNA in stepwise manner by removal of 5' mononucleotide units from the end that bears an unesterified 3' hydroxyl group, 90% of the hyperchromicity is lost (56). In contrast, when sRNA was hydrolyzed with 0·5 N KOH where attack is known to be random and endonucleolytic, the increase in optical density roughly paralleled the degree of hydrolysis. Cantoni et al (56) have concluded from these results that sRNA contains only one long base-paired structure and proposed a model (see Fig. 1A) which appears to be rigid, rodlet-shaped and folded back on itself by hydrogen bonding like a hair-pin structure.

The melting curves obtained with yeast sRNA in the presence of Mg^{++} ions show a sharply defined transition. The melting profiles obtained in the absence of Mg^{++} ions show an extremely

Fig. 1



Two possible models for sRNA. The amino acid acceptor end is indicated by a solid circle

broad transition ranging from below room temperature to about 90°C (48). Mahler et al (58) have found that in the presence of Mg^{++} ions different fractions of sRNA melt at different temperatures and that the variation between fractions follow their guanine and cytosine content in a manner analogous to that observed with DNA. These observations also suggest that the sRNA molecules are capable of forming long helical structure with complementary base-pairing. The work of Spencer showed that the melting curves obtained with 2·5 S material in the presence of Mg^{++} ions do not show the sharp transition found with 4 S material, the transition with 2·5 S material is almost as broad as in the absence of Mg^{++} ions. This observation correlates with those of Monier et al (59). They observed an irreversible change in the melting curve of sRNA after heating. The melting curve obtained on heating was typical of 4 S material. These observations suggested an alternative model (see Fig. 1B) for the sRNA molecule (53). In this model there are two hair-pin like regions in a 4 S material and they are linked by a single-stranded region. This region may introduce some constraint which could prevent regular crystalline arrangement. Heating might lead to separation of these two helical regions so that they could arrange themselves freely in a crystalline lattice.

Chemical properties of sRNA

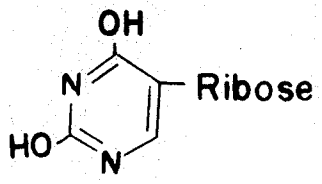
The ester linkage of the sRNA with the amino acid appears to be relatively unstable especially at neutral or alkaline pH. The unusual reactivity of the ester linkage towards nucleophilic reagents as well as its high free energy of hydrolysis (25, 32, 60)

have been shown to arise mainly from the influence of the positively charged α-amino group of the esterified amino acid at neutral pH (60). The protonated α-amino group greatly enhances the rate of attack by nucleophilic reagents and reduces the thermodynamic stability of amino acid esters. Wolfenden (60) observed that throughout the pH range studied hydroxyl ions attack leucyl RNA thirty times more rapidly than leucine ethyl ester. This difference in rate of hydrolysis can be mainly attributed to the presence of a cis-hydroxyl group on the ribose adjacent to that on which the amino acid is esterified. The size and the chemical structure of the amino acid side chain have also been shown to have influence on the stability of the amino acyl RNAs (61, 109).

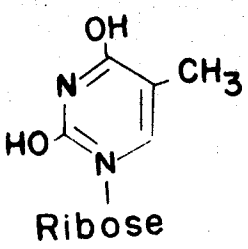
sRNA preparations from various sources are similar in their high guanine (about 32-35%, see Table 1) and cytosine (28-32%) content and the near equivalence of complementary bases. The finding of an equivalence of bases supports the idea of base-pairing in the molecule. sRNA regardless of the tissue source has been found to contain a number of odd nucleotides not found to any appreciable extent in other types of RNA. Pseudouridylic acid has been reported to constitute about 25% of the UMP in rat liver sRNA (62, 63). Methylated purines and pyrimidines amount to 2% of the bases in sRNA (64). The following methylated bases have been found in sRNA from different sources (see Fig. 2): 6-amino-2-methyl-purine (64, 65), 6-methyl-amino-purine (64, 66), 6-dimethyl-amino-purine (64, 65), 6-amino-1-methyl-purine (66), 2-amino-6-hydroxyl-1-methyl-purine (62, 64), 6-hydroxyl-2-methyl-amino-purine (64-66), 6-hydroxyl-2-dimethylamino-purine (64-66), 6-hydroxyl-1-methyl-purine (67), ribosyl thymine (62, 65) and

Fig. 2

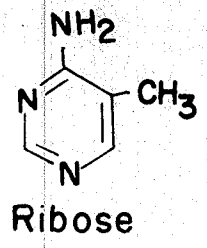
MINOR COMPONENTS FOUND IN sRNA



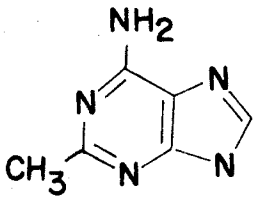
Pseudouridine



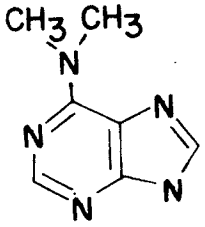
Ribosyl thymine



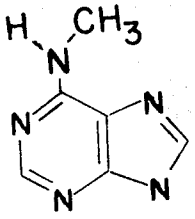
5-Methyl cytosine



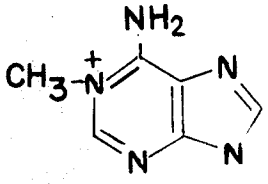
6-Amino 2-methyl purine



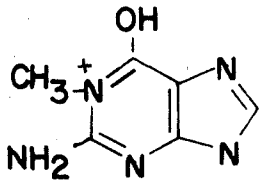
6-Dimethylamino purine



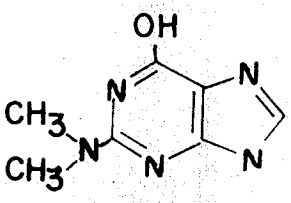
6-Methylamino purine



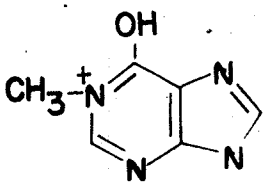
6-Amino l methyl purine



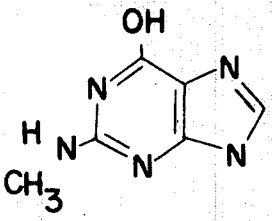
2-Amino 6-hydroxy l-methylpurine



6-Hydroxy 2-dimethylamino purine



l-Methyl 6-hydroxypurine



6-Hydroxy 2-methylaminopurine

5-methyl-cytosine (68, 69). The idea that different amino acid acceptor RNAs have different amounts of odd bases is indicated by the fact that yeast tyrosyl RNA, separated by Holley et al (43) have been shown to contain a total of nine different methylated bases whereas alanyl- and valyl RNAs contain only five methylated bases.

Only one mole of guanosine diphosphate (pGp) and one mole of adenosine are released per mole of liver sRNA on alkaline digestion (70, 74). This finding supports the ideathat the sRNA molecule consists of a single-stranded poly nucleotide chain. Although recently Bell, Tomlinson and Tener (71) and Zillig et al (72) with yeast sRNA preparations have obtained evidence for the existence of pUp..., (10%), pAp..., (7%) and pCp..., (5%), they found pGp..., (78%) by far the most common 5' terminal moiety for the average sRNA chain. Other investigators have found only pGp. Further information about the sequence for the acceptor end was obtained from studies of incorporation of α-P^{32} labelled CTP and ATP into enzymatically pyrophosphorylzed sRNA. The results revealed that two moles of CMP were incorporated per mole of AMP (33). Upon alkaline digestion of P^{32} CTP labelled sRNA about 50% of the P^{32} was found in the form of CMP and the remaining 50% was distributed in other nucleotides. These findings strongly suggest that most sRNA molecules have identical ends corresponding to the sequence pGp...pCpCpA (67). These terminal sequences for unfractionated sRNA chains have been found also in sequence studies with partially purified sRNAs, namely, the yeast alanyl-, valyl- and tyrosyl RNAs separated by Holley et al (43), the valyl RNA separated by Ingram

et al (73) and the seryl-RNA separated by Cantoni et al (22).

The results of these sequence studies also indicate that the nucleotide compositions of these sRNAs are all significantly different from one another. The two dimensional chromatographic patterns for pancreatic RNase (43, 73) and T-1 RNase (73) digests of these sRNAs establish that the nucleotide sequences of the four sRNAs are also very different. The internal sequences of nucleotides in the specific acceptor RNAs must therefore be different even though the terminal sequences are identical. Berg et al (50) have shown heterogeneity in the nucleotide sequences adjacent to the terminal acceptor ends. By using specific degradation techniques they reported that 69% of the sRNA chains contain adenine and 17% contain guanine in the fourth position and 94% contain a pyrimidine in the fifth position. Studies on specific sRNAs for leucine and isoleucine show the following terminal sequences (57):

leucine-1	.....GpCpApCpCpA
leucine-2	.....GpUpApCpCpA
isoleucine	.....GpCp(UpC)pApCpCpA

These results clearly demonstrate that specific sRNAs differ from each other by different nucleotide sequences between the two ends.

Nihei and Cantoni (75) in studies of the hydrolysis of sRNA with snake venom phosphodiesterase claim to have determined the proportion of minor components occurring in the acceptor end, the center and the non-acceptor end and concluded that the minor components are concentrated in the center of the poly nucleotide chain. However, Holley et al (67) have presented the

nucleotide sequence of yeast alanyl RNA showing that the minor bases also occur in the two limbs, as well as in the center, of the polynucleotide chain.

The recent observation of Bell et al (76) on the non-acceptor end of yeast sRNA indicates that the sequence of pGpCp... is the predominant sequence in average sRNA molecules. It can be seen from the two terminal nucleotide sequences of sRNA as reported by Bell et al (76) and Berg et al (50) that the single hair-pin structure of sRNA as proposed by Cantoni et al (56) (see Fig. 1A) would not fit with these observations on terminal sequence studies. The cytosine in the sequence of pGpCp... cannot base pair with the fourth nucleotide in acceptor end if the fourth base is predominantly adenine as found by Berg et al (50).

There are probably three specificity sites on sRNA molecules i) the site with which the amino acyl synthetase interacts to esterify the sRNA with the amino acid, ii) the site, known as the coding region, which interacts with the template and iii) the site which provides the species specificity for the attachment of the specific amino acyl synthetase to the sRNA. Hecht et al (77, 78) showed that the acceptor activity of liver sRNA required the intactness of a specific terminal sequence of nucleotides ...pCpCpA. The leucyl RNA and phenylalanyl RNA synthetases from E. coli showed no measurable activity with yeast sRNA, whereas the valyl RNA- and phenylalanyl RNA synthetases from yeast were able to esterify E. coli sRNAs to approximately the same extent as do the homologous enzymes (79). There is additional evidence to support the hypothesis of separate specificity sites. It

has been shown in the case of E. coli phenylalanyl- and lysyl RNAs that the ability of the sRNA to interact with the microsomes and to transfer the amino acids into polypeptide chains can be destroyed by deamination of the bases with nitrous acid without affecting the ability to accept amino acid (80). Ebel et al (81) in studies of methylation and bromination of yeast sRNA have also concluded that in the sRNA molecule the sites responsible for the accepting capacity are different from the sites responsible for the transferring capacity.

Purification of specific acceptor RNAs

Two general types of fractionation methods for amino acid acceptor RNA have been used: i) physicochemical methods, such as absorption and elution from chromatographic columns, electrophoresis and counter current distribution; ii) chemical methods, depending on covalent bonding to the sRNA of substances which modify the properties of the sRNA and thus make the separation possible.

Stephenson and Zamecnik (82, 83) have obtained about 90% pure valyl RNA from yeast by using combined chromatographic and chemical procedures. The amino acids which were bound to the unfractionated sRNA were removed by hydrolysis by incubation at pH 10. After removal of all bound amino acids and labelling the sRNA with only valine the free adjacent 2' and 3' hydroxyl groups on the terminal ribosyl moiety of the sRNA molecules which did not carry bound valine were oxidised to the corresponding dialdehyde by periodate (45, 83). The dialdehyde derivatives of sRNA were then reacted with 2-hydroxy 3-naphthoic acid

hydrazide to form a hydrazone. This colourless hydrazone was in turn reacted with tetrazotized O-dianisidine with the formation of a deep blue dye, bound to the sRNA. Thus amino acyl RNA present in the mixture did not form a complex with the dye. The physical properties of the dye-bound sRNA and amino acyl RNA differ sufficiently to allow partial separation of amino acyl RNA. The mixture was chromatographed on DEAE-Sephadex at pH 6·0. Most of the dye-bound RNA was retained on the column while the valyl RNA was eluted when the proper salt gradient was used. It was subsequently found, however, that not all species of amino acyl RNA emerge before the bulk of the dye-bound RNA. This was indicated from experiments with tyrosyl-RNA and seryl-RNA. Leucyl RNA has been partially purified (70%) from E. coli by Zubay (45). This investigator used the same method employed by Zamecnik et al outlined above except that the Schiff base was formed between the sRNA dialdehyde and a primary amine, aminoethyl cellulose, in the form of a chromatographic column. This result has not been extended further by Zubay probably because of some difficulty in purification, as a result of β-elimination. This reaction involves the cleavage of the adenosine terminal group from the remainder of the sRNA chain. The reaction depends upon the presence of a double bond in the position β to the phosphorus group and results in the cleavage of a P-O bond (76). As a result of this cleavage, the bulk of the sRNA is left with a terminal 3' phosphate group and is released from the column.

Fractionation of sRNA by chromatography on Cato-3 starch

column has been reported by Smith et al (85). They observed relative enrichment of the tyrosyl RNA in eluates of low salt concentration and of leucyl RNA in eluates of higher salt concentration. Berg (28) has performed chromatography of E. coli sRNA on 3- to 12-foot high columns of dextren gel, using ethoxyethanol, butoxyethanol and triethanolamine. Enrichment of acceptor activity with this method ranged from 3- to 17-fold for several amino acid acceptor RNAs examined (theoretically, the limit of purification of sRNA is 20-fold which is based on the assumption that there are 20 different sRNAs and/are that they each present in an equal amount).

Of the many methods in the literature which have been used for purifying amino acid acceptor RNA, counter current distribution is considered to be the most successful and reliable method. It gives a relatively high purity as well as reproducible results. Holley et al (86) have obtained purified alanyl- (66%), tyrosyl- (45%) and valyl- (60%) acceptor RNAs from yeast by the counter current distribution technique with a solvent system containing formamide and isopropanol in phosphate buffer (43, 86).

Partition chromatography, which utilizes the same principle as counter current distribution, has been used for the fractionation of sRNA. Everett et al (87) reported that silica gel is not an ideal supporting medium for partition chromatography of sRNA. Cantoni et al (21) have performed partition chromatography of yeast sRNA on Sephadex G25 column, which offers theoretical advantages, being more hydrophilic than silica gel. Enrichment of up to 2-fold for tyrosyl RNA and 10- to 12-fold

for seryl-RNA have been reported.

It is of interest to note that the partition coefficients of the different sRNAs extend over a 36-fold range in some solvent systems (86). This wide range of partition coefficients of sRNA may be considered as an indication that different sRNAs have different base ratios. Presumably each has a characteristic guanine-cytosine content. Many physical properties of sRNA, of DNA, as well as of other types of RNA, are related to their GC-content (89). Other factors, such as differing amounts of methylated bases and pseudo-UMP, probably also contribute to the difference in physical properties among different sRNAs on which separation of sRNA by counter current distribution depends.

Methylated albumin column has been demonstrated to separate sRNA from 16 S and 23 S ribosomal RNA (90, 91). The investigations of Sueoka and Yamane (92) show elution profiles from methylated albumin columns for 16 amino acid acceptor RNAs. Each has a characteristic elution pattern, and multiple components are observed, most prominently with leucine, isoleucine and tryptophan.

The selective precipitation of sRNA bearing an amino acid by building up a synthetic polymer on the free amino group of the bound amino acid has been demonstrated with the N-carboxy anhydrides of several amino acids, including phenylalanine and tryptophan. However, biologically active sRNA was not obtained after any treatment that was capable of solubilizing the polymer

formed with any of these reagents. Mehler et al (93) have carried out the polymerisation of the N-carboxy anhydride of ∈-trifluoroacetyllysine (79) with sRNA charged only with a particular amino acid, since the trifluoroacetyl group had been shown by Goldberger and Anfinsen (84) to be hydrolysed under very mild alkaline conditions. The procedure involves removal of all bound amino acids and recharging the sRNA with a particular amino acid enzymatically. This is followed by precipitation of the specific sRNA after reaction with N-carboxy anhydride of ∈-trifluoroacetyl lysine. This reaction forms an insoluble polymer. The ester bond between the polypeptide and the sRNA is then hydrolysed to release the purified sRNA. The specific sRNAs recovered from the polymer are reported to be enriched 3·2-fold for both valyl- and leucyl-RNAs.

Only a few experiments (61) have been made up to the present time which have yielded some information on glycine acceptor RNA and will be referred to later in the Discussion.

Determination of structure of sRNA

Elucidation of the intimate structure of various nucleic acids constitutes one of the long range objectives in nucleic acid research. sRNA is a particular object for this type of study because of its relatively short chain length and its unique ability to function as an adaptor in protein biosynthesis.

The principle used in determination of the base sequence in sRNA is analogous to the one Sanger (88) had used to elucidate the complete sequence of amino acids in insulin molecule. However, the task of fitting together the various nucleotide sequence

components of sRNA becomes very much more difficult owing to the fact that not only the problem of separation of a desired amino acyl RNA is still an impediment to progress, there are only four major different building blocks in sRNA compared to 20 different amino acids in protein. However, the minor nucleotides in sRNA molecules may provide suitable markers in sequence studies.

Various approaches have been made to the problem of the determination of nucleotide sequences in sRNA. The employment of all available enzymes which attack sRNA, no matter whether they are used singly or in combination, has not yet permitted an elucidation of the complete sequence of sRNA. One approach has been to cleave the sRNA molecule first at as few specific points as possible to give relatively large fragments. It is known, for example, that RNase T1 splits all the internucleotide bonds between guanylic acid and adjacent nucleotide in sRNA, while almost all other bonds are not affected (94). Its digestion products contain relatively large fragments of oligonucleotide terminated with 3' guanylic acid together with guanylic 3'monophosphate. Identification of the sequences in oligonucleotides obtained from RNase T1 digests can easily be achieved but the overlapping sequences in sRNA remain unknown. One could not be sure, for example, whether a partial sequence is GGGACUG or GGACUGG. If the partial sequences obtained by the action of RNase T1 compared with those from pancreatic RNase, overlapping fragments can result and thus it should give a more detailed sequence in sRNA molecule.

Degradation from one end of the sRNA chain can be achieved

by digestion with snake venom phosphodiesterase or by repeated removal of the terminal nucleotide by periodate oxidation followed by β-elimination reaction or alkaline destruction (95) of the oxidized terminal residue. The appearance of a particular odd base(s) in a given digestion time, in the case of digestion with snake venom phosphodiesterase, can give the relative position in the sRNA polynucleotide chain of the oligonucleotide fragments obtained by digesting with RNase T1 which contain that odd base. This technique of degradation from one end of the sRNA chain can also be used to obtain a partially degraded sRNA for sequence studies.

Another approach has been the modification by various chemical methods of the point of attack of the enzyme. This method also yields overlapping oligonucleotides. A number of chemical modifications have been developed in the past few years. Zillig et al (96) have shown that it is possible to modify CMP or UMP of the sRNA selectively with hydroxylamine by controlling the pH. The pH optimum for the reaction with UMP lies at pH 10·1 whereas that for CMP is 6·1. No reaction was found in the case of adenine and its derivatives, whereas those of guanine and thymine reacted only very slowly under the conditions used. Pancreatic RNase was found not to attack the chain at the sites where the bases had been modified. The result of this chemical treatment is to alter the point of attack by pancreatic RNase yielding larger oligonucleotide fragments containing ACG with U as 3' terminal in one case, or AUG with C as 3' terminal in other case. It has also been found that in acid solution bromine

reacts rapidly with UMP and CMP but more slowly with GMP. AMP does not react with bromine under these conditions. Nitrous acid is known to cause deamination of the bases (80). These chemical modifications of sRNA prevent the attack of RNases at certain positions which would be normally attacked by the enzymes. The digestion then yields different oligonucleotide fragments. Thus, means are now available, by combining the chemical modifications and the digestions by RNases techniques, for the elucidation of complete nucleotide sequences of sRNA molecules.

Purpose of thesis

The purpose of this investigation was to study the chemical properties of glycyl RNA of rat liver. Attempts were first made to separate glycyl RNA from rat liver, since a single molecular type of pure sRNA specific for a particular amino acid is needed for most physical and chemical studies. Attempts have also been made to find methods for the stabilization of the ester linkage of glycyl RNA which, as demonstrated by Meister et al (61), is the most unstable of all amino acyl RNAs. Acetylation of glycyl-RNA has been found to produce an ester with a half-life about ten times longer than that of glycyl RNA without affecting the acceptor capacity of the sRNA moiety. The terminal nucleotide sequence at the acceptor end of rat liver glycyl RNA has been shown by direct enzymatic and chemical methods, to be pPypCpA. This is in agreement with the terminal pCpCpA sequences determined for some other acceptor RNAs.

Radioisotopes

Six preparations of 1-C^{14}-glycine were used in this work. One preparation was obtained from the New England Nuclear Corporation, Boston 18, Massachusetts, U.S.A. and had a specific activity of 77 μc/mg; 1 c.p.m. was equivalent to 0·195 μμmoles of glycine. Another preparation was obtained from the Schwarz Bioresearch Inc., Mount Vernon, New York, U.S.A. and had a specific activity of 10·5 μc/mg; 1 c.p.m. was equivalent to 2·14 μμmoles of glycine. Others were all obtained from Merck Sharp and Dohme of Canada Ltd., Montreal, Canada and had the specific activities of 22·5 μc/mg; 1 c.p.m. = 0·69 μμmoles of glycine; 22·5 μc/mg, 1 c.p.m. = 0·86 μμmoles of glycine; 22·5 μc/mg, 1 c.p.m. = 0·91 μμmoles of glycine and 19·8 μc/mg, 1 c.p.m. = 0·95 μμmoles of glycine, respectively. The radioactive glycine samples had been found to be chromatographically pure.

Chemicals

Nucleoside triphosphates were purchased from the California Corporation for Biochemical Research, Los Angeles 63, California, U.S.A.

Amino-ethyl cellulose, AE-50, Whatman anion exchanger, was obtained from W. & R. Balston Ltd., England.

Crystalline pancreatic RNase was obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

All other chemicals were "Analar" grade, obtained from the British Drug Houses (Canada) Ltd., Toronto, Ontario.

Tissue fractions

All experimental results were obtained using fresh rat livers of 150-200 gm male Holtzman albino rats, purchased from the Holtzman Co., 421 Holtzman Road, Madison, Wisc., U.S.A.

After the rats had been stunned by a blow on the head the livers were quickly removed and dropped into ice-cold 0·35 M sucrose solution. All procedures followed thereafter were carried out in the cold unless otherwise stated. Fat and connective tissues were dissected from the livers and then they were weighed and cut into small pieces with scissors. The pH 5 enzyme fraction was prepared in a manner similar to that of Keller and Zamecnik (98). "Liver medium A" (containing 0·35 M sucrose, 0·025 M KCl, 0·01 M $MgCl_2$ and 0·017 M K_2HPO_4, pH 7·5, adjusted by the addition of 1 N HCl) was added to the minced liver in a volume equivalent to 2·3 times the weight of the fresh liver used. The preparation was then homogenized quickly by making 6 to 7 passes with a Potter homogenizer. The liver homogenate was centrifuged at 10,000 r.p.m. for 10 minutes in a Servall RC-2 refrigerated automatic centrifuge. The supernatant was then placed in a Spinco model L refrigerated ultracentrifuge and spun at 105,000 x g for one hour. The "105,000 x g supernatant" was carefully removed and the pH adjusted to 5·1-5·2 by the dropwise addition of 1 N acetic acid. The mixture was allowed to stand in ice for about 5 minutes to allow complete precipitation of the "pH 5 enzymes". The mixture was then centrifuged at 10,000 r.p.m. for 10 minutes and the supernatant was discarded. The precipitate was suspended in a volume of medium A equal to the weight of the liver used. The supernatant was adjusted to pH 7·5

by dropwise addition of 1 N KOH. At this stage the preparation is known as the "pH 5 enzymes" fraction.

Preparation of partially purified glycyl RNA synthetase

In most preparations 20 mls of the freshly prepared "pH 5 enzymes" fraction were used to prepare glycyl RNA synthetase according to the method of Fraser (23). It was found that the "pH 5 enzymes" fraction could be frozen at -20°C for about two weeks with little or no loss of glycyl RNA synthetase activity. To 20 mls of the fresh preparation, 0·4 mls 0·05 M ATP, pH 7·5 were added. The mixture was heated at 55°C for 3 minutes and then cooled in an ice bath. The heated pH 5 enzymes were centrifuged at 10,000 r.p.m. for 10 minutes and the precipitate was discarded. The supernatant was made 50% saturated with respect to $(NH_4)_2SO_4$ by the slow addition of solid $(NH_4)_2SO_4$ (0·2974 gm per ml supernatant) without adjusting the pH. The mixture was gently stirred with a magnetic stirrer in the cold for 15 minutes. The mixture was then centrifuged at 10,000 r.p.m. for 10 minutes and the precipitate was discarded. The supernatant was then made 60% saturated by the addition of more solid $(NH_4)_2SO_4$ (0·0627 gm/ml) in a similar manner. After gentle stirring in the cold for 15 minutes, the mixture was centrifuged at 10,000 r.p.m. for 15 minutes and the supernatant was discarded. The resulting small precipitate was then dissolved in a volume of 0·1 M Tris-HCl buffer pH 7·5 equivalent to one-tenth the volume of the "pH 5 enzymes" fraction used for its preparation. This fraction contained glycyl RNA synthetase and was called "50-60% $(NH_4)_2SO_4$". It contained about 0·13-0·18 mg sRNA and 1·5-1·8 mg protein per

10 ml pH 5 enzymes originally used.

Isolation of sRNA from rat liver pH 5 enzymes fraction

The isolation procedure of Kirby (99) was followed. An equal volume of 90% w/v phenol was added to the "pH 5 enzymes" fraction. The mixture was shaken mechanically for one hour and then centrifuged at 10,000 r.p.m. for 20 minutes. The top aqueous layer was carefully removed with a Pasteur pipette without disturbing the denatured protein in the interface. The aqueous solution was extracted twice with an equal volume of ether to remove traces of phenol. Then 0·1 volume of 20% K-acetate pH 5 was added, followed by the addition of 2·5 volumes of 95% ethanol (-20°C) to precipitate the sRNA. The mixture was kept in the deep freeze for at least 4 hours. It was found that the sRNA prepared by this procedure could be kept in the deep freeze in the alcoholic suspension for at least 3 months without loss in glycine acceptor activity. The sRNA was subsequently washed twice with 95% ethanol before use. This sRNA preparation is referred to as "phenol extracted sRNA".

Some preparations of sRNA were further purified by the following treatment. sRNA obtained from the above procedure was dissolved in distilled water (about 20 mg sRNA in 10 mls of H_2O) and solid NaCl was added to make the final concentration of 1·0 M NaCl. After stirring in the cold for 5-6 hours the mixture was centrifuged at 7,000 r.p.m. for 15 minutes and the precipitate was discarded. The supernatant was dialysed overnight against two changes of 5 litres of distilled water. The dialysate was mixed with an equal volume of 2·5 M K-phosphate buffer, pH 7·0, then

a third volume of methyl cellosolve was added. The resulting mixture was stirred for 5 minutes at room temperature and then centrifuged at 10,000 r.p.m. for 10 minutes. The upper layer was removed with a Pasteur pipette and to it was added 0·1 volume of 20% K-phosphate pH 5·0 and 2·5 volume of cold 95% ethanol to re-precipitate the sRNA. After standing in the deep freeze for about 4 hours the precipitate was collected by centrifugation. It was dissolved in a minimum amount of glass distilled water, and dialyzed against two changes of distilled water. The sRNA was recovered from the dialysate by the acetate-ethanol precipitation procedure outlined above. This sRNA preparation is referred to as "purified sRNA".

Determination of base composition of unfractionated rat liver sRNA

Phenol extracted sRNA was hydrolyzed with 0·4 N KOH at 37°C for 18 hours. The hydrolysis was stopped by the addition of 1 N $HClO_4$ lowering the pH to 3·5. The resulting mixture was kept in the cold for about 15 minutes to allow precipitation of the excess of K-perchlorate and the precipitates were removed by centrifugation.

The separation of nucleotides of sRNA hydrolysates was achieved by chromatography on Dowex 1-Cl^- column using stepwise elution of the absorbed nucleotides. Mixtures of 2' and 3' CMP, AMP, UMP and GMP were eluted with 0·001 N, 0·0022 N, 0·003 N and 0·0055 N HCl respectively.

Paper-strip electrophoresis was also used. Small amounts of the sRNA alkaline hydrolysate (about 2·0 optical density units measured at 260 mμ) containing a mixture of 2' and 3'

nucleotides were applied to paper strips by the "dry loading" method. The electrophoresis was carried out in a Spinco model R paper electrophoresis cell, Durrum type, in 0·02 M Na-citrate buffer pH 3·5 by the application of a constant potential of 500 volts. The current drawn was about 50 mA. The electrophoresis was performed at room temperature for 3 hours. Blank paper strips were run simultaneously as controls. The U.V. absorbing regions, which were detected under the U.V. lamp (Ultra Violet Inc., San Gabriel, Calif., U.S.A.) and the corresponding blank regions were cut from the papers, and eluted with 0·01 N HCl to the desired volume. Optical densities and absorption spectra of the eluates were measured with the Beckman DU spectrophotometer.

Method for assaying the acceptor activity of sRNA

The acceptor activity of the sRNA was tested by measuring the incorporation of 1-C^{14}-glycine into sRNA catalyzed by glycyl-RNA synthetase either in the form of "pH 5 enzymes" fraction or in the form of partially purified "50-60% $(NH_4)_2SO_4$" fraction (23). The incubation mixture contained, in a final volume of 1·0 ml, 10 µmoles $MgCl_2$, 10 µmoles mercaptoethanol, about 2·5 mg serum albumin previously run through a Sephadex column, 5 µmoles ATP, pH 7·5, 60 µmoles Tris-HCl buffer pH 7·5, about 0·15 µmoles 1-C^{14}-glycine, 0·1 ml 50-60% $(NH_4)_2SO_4$ fraction (or pH 5 enzymes) and various amounts of sRNA. The enzyme preparation was added last at "zero time" to start reaction. The incubation was carried out at 37°C for 10 minutes. The samples were precipitated and washed according to the method of Berg et al (50). About 1·5-2·0 mg of yeast RNA carrier were added to each reaction mixture, followed quickly by 3·0 volumes of cold 0·5 M NaCl-67% ethanol mixture to

stop reaction. The mixtures were chilled in the deep freeze for about 20 minutes, centrifuged and then washed 3 times with the same salt ethanol mixture. The final precipitates were each dissolved in 1·0 ml of 1·5 M NH_4OH, plated on clean planchets and counted after the samples had been dried under an infra-red lamp.

Another washing procedure (23) was also used. The reactions were stopped by the addition to each sample of 1·0 ml of cold 22·5% TCA containing 2% C^{12}-glycine as carrier. A suitable amount of heat-denatured (75°C, 3 mins.) pH 5 enzymes was added to each sample as protein-nucleic acid carrier. The precipitates were spun down in a clinical centrifuge in the cold for 3 minutes. The precipitates were then washed twice with 5·6% TCA with 2% glycine carrier, twice with cold acetone and once with ether. Finally, the precipitates were suspended in 1·0 ml of ether and plated on clean, lightly greased planchets and counted.

All radioactive samples were counted at infinite thinness usually for 10 minutes on a Nuclear Chicago model 181 A micromil window gas-flow counter. Samples were run in duplicate. The counts were corrected for background. The incorporations are expressed as μμMoles of glycine per mg of sRNA.

Preparation of amino-ethyl (AE-50) cellulose column

A suitable amount of AE-50 cellulose (45) was suspended in 0·01 N NaOH and poured into a column of 1 cm in diameter. The column was then washed successively with 500 mls 0·01 N NaOH, 500 mls distilled water, 500 mls 0·01 N HCl, and finally with 500 mls distilled water again. The column was then allowed to

equilibrate with the appropriate buffer before the sRNA sample was applied to the column in the buffer solution.

Preparation of oxidized and 1-C^{14}-glycine labelled sRNA

Phenol extracted sRNA was incubated with 0·1 N Na-bicarbonate buffer pH 10 at 37°C for 30 minutes to strip off all bound amino acids from sRNA. After incubation the stripped sRNA was recovered by the acetate-ethanol precipitation procedure. The stripped sRNA was then labelled with 1-C^{14}-glycine by the method described above. The mixture of labelled and stripped sRNAs was re-isolated by the phenol method. Then it was dissolved in 0·1 M Na-acetate buffer pH 5·0 and an equal volume of 0·2 M Na-periodate (45) in 0·1 M Na-acetate buffer was added and the resulting mixture was allowed to stand at room temperature for 30 minutes, after which solid NaCl was added (about 80 mg/ml) to the mixture and the sRNA was recovered by the addition of 2·5 volumes of 95% ethanol and centrifuging. The sRNA precipitates were further washed 2 or 3 times with 80% ethanol.

Acetylation of 1-C^{14}-glycine labelled sRNA

The stripped sRNA was labelled with 1-C^{14}-glycine enzymatically and was re-isolated by the phenol method. The labelled sRNA (free of amino acids other than glycine) was washed 3 or 4 times with acetate-ethanol mixture (20% K-acetate pH 5·0 : 95% ethanol = 1:2·5) and then it was transferred to a clinical centrifuge tube and washed once with 95% ethanol. The inside wall of the centrifuge tube was swabbed dry with a piece of tissue paper. About 5-10 mls of glacial acetic acid was added to the labelled sRNA sample to make a fine suspension (100). It was then transferred to a suitable

flask and heated to 55°-60°C for 3 minutes (taking care to avoid over-heating). After cooling down to room temperature an equal volume of acetic anhydride was added dropwise and again heated to 55°-60°C for 3 minutes. Then the mixture was brought to room temperature and the sRNA re-precipitated by the acetate-ethanol procedure.

After washing twice with 95% ethanol the acetylated 1-C^{14}-glycine-labelled sRNA was then dissolved up in 0·25 M Tris-buffer pH 7·5 and incubated at 37°C, withdrawing aliquots at various time intervals. Duplicate aliquots were removed from the bulk incubation mixture and the sRNA precipitated by the addition of 0·1 volume of 20% K-acetate pH 5·0 and 2·5 volume 95% cold ethanol. These samples were kept in the deep freeze for about 20 minutes, then the sRNA was recovered by centrifugation. The sediments were each dissolved in 0·8 mls of water, plated on stainless steel planchets and counted. For comparison an un-acetylated 1-C^{14}-glycine-labelled sRNA was carried through the same process simultaneously.

Reconstitution of glycine acceptor activity of RNase treated sRNA

Phenol extracted sRNA was dissolved in distilled water and various small amounts of pancreatic RNase were added in different experiments. The mixture(s) was incubated at room temperature in a water bath for 10 minutes (101). The reaction was stopped by the addition of an equal volume of 90% w/v phenol. A control was run simultaneously by shaking the sRNA solution briefly with phenol before the addition of RNase solution then continuing the shaking with phenol after the addition of RNase solution.

Reconstitution (102) of pancreatic RNase treated sRNA was carried out as follows: samples of the RNase treated sRNA

were pre-incubated with 0·1 ml pH 5 enzymes fraction at 37°C for 20 minutes in the absence or in the presence of different nucleoside triphosphates (1·0 µmole/ml incubation mixture) together with 10 µmoles $MgCl_2$, 10 µmoles mercaptoethenol, 5 µmoles ATP, pH 7·5 and 50 µmoles Tris buffer, pH 7·5. Then, after 20 minutes for pre-incubation, 0·05 mls 12·9 mM 1-C^{14}-glycine were added to each reaction mixture to make a final volume of 1·0 ml. The mixtures were incubated for a further 10 minutes. After incubation, the samples were washed according to the washing procedure of Berg et al as described previously.

Pyrophosphorolysis and reconstitution of sRNA

An appropriate amount of pH 5 enzymes fraction dissolved in medium A was made 4·5 µmoles/ml in KF (103, 104) and 4·5 µmoles/ml in inorganic pyrophosphate. The resulting mixture was incubated at 37°C for 30 minutes to permit pyrophosphorolysis (74, 105). Then the pyrophosphorolyzed sRNA was isolated from the reaction mixture by the phenol method.

The conditions for reconstitution (102) of the glycine acceptor activity of pyrophosphorolyzed sRNA were essentially the same as that described above for RNase treated sRNA. After incubation the reactions were stopped by the addition of 1·0 ml 22·5% TCA containing 2% glycine carrier and the samples were washed as described above.

Finger-print of pancreatic RNase digest of acetylated 1-C^{14}-glycine labelled sRNA

The stripped sRNA was labelled enzymatically with 1-C^{14}-glycine

and subsequently acetylated as described above. Then the acetylated glycyl RNA preparation was dissolved in 1 to 2 mls of distilled water, bringing the pH to about 6·5. A small amount of pancreatic RNase solution was added to the sRNA solution to a final concentration of 250 µg RNase per ml. The mixture was incubated at room temperature in a water bath for 10 minutes. Then 0·32 mls 1 N PCA/ml was added to stop reaction. The small precipitate that formed was removed by centrifugation. To the supernatant 1 N KOH was carefully added dropwise to bring the pH to 3-4 (taking care to avoid too much alkali). The small precipitate that formed was removed and usually 0·1 ml of the supernatant was applied to an 46·3 x 57 cm^2 paper sheet which was just wet with ammonium formate buffer pH 2·7 (106). After loading the sample, the paper was quickly hung on a special rack and placed immediately in a high voltage electrophorator model D (Gilson Medical Electronics, Middleton, Wisc., U.S.A.). The electrophoresis was carried out at a constant voltage of 1,500 volts at room temperature with water cooling for one hour. The current drawn was about 50 mA.

After electrophoresis the paper sheet was lifted out of the electrophorator and dried in air. The dried paper sheet was then subjected to one-dimensional chromatography. The solvent system (107) consisted of 100 mls tertiary butanol, 99·7 mls distilled water and 0·3 mls 98% formic acid, and had a pH of 3·6 (adjusted by the addition of conc. ammonia). The direction of chromatography was at right angles to the direction of electrophoresis. The chromatography was allowed to proceed at room temperature for about 16 hours (over-night). The paper sheet was

then removed from the chromatographic tank and dried in air. The U.V. absorbing spots were detected under a U.V. lamp. In control finger-prints, free N-acetyl glycine was detected by spraying with bromophenol-blue (50 mgs bromophenol blue in 100 mls water containing 200 mgs citric acid), free glycine was detected with the ninhydrin reagent (Sigma Chemical Co.). Optical densities and absorption spectra of the spots were measured with Beckman DU spectrophotometer. The radioactivities of the spots were determined by eluting the spots with 0·01 N HCl, plating on planchets, drying down and counting.

Analytical determinations

sRNA was estimated by measuring the absorption at 260 mμ using 1 mg = 20 O.D. units at 260 mμ/ml as the extinction coefficient in the calculation (108).

Protein estimations were made by the method of Lowry et al (109), using 5 x crystallyzed egg albumin (Sigma Chemical Co.) as a standard.

RESULTS

Determination of base composition of sRNA

The results of determinations of base composition of unfractionated sRNA from rat liver by both paper-strip electrophoresis and chromatography on Dowex 1-Cl^- column are presented in Table 1. Literature values are also listed for comparison.

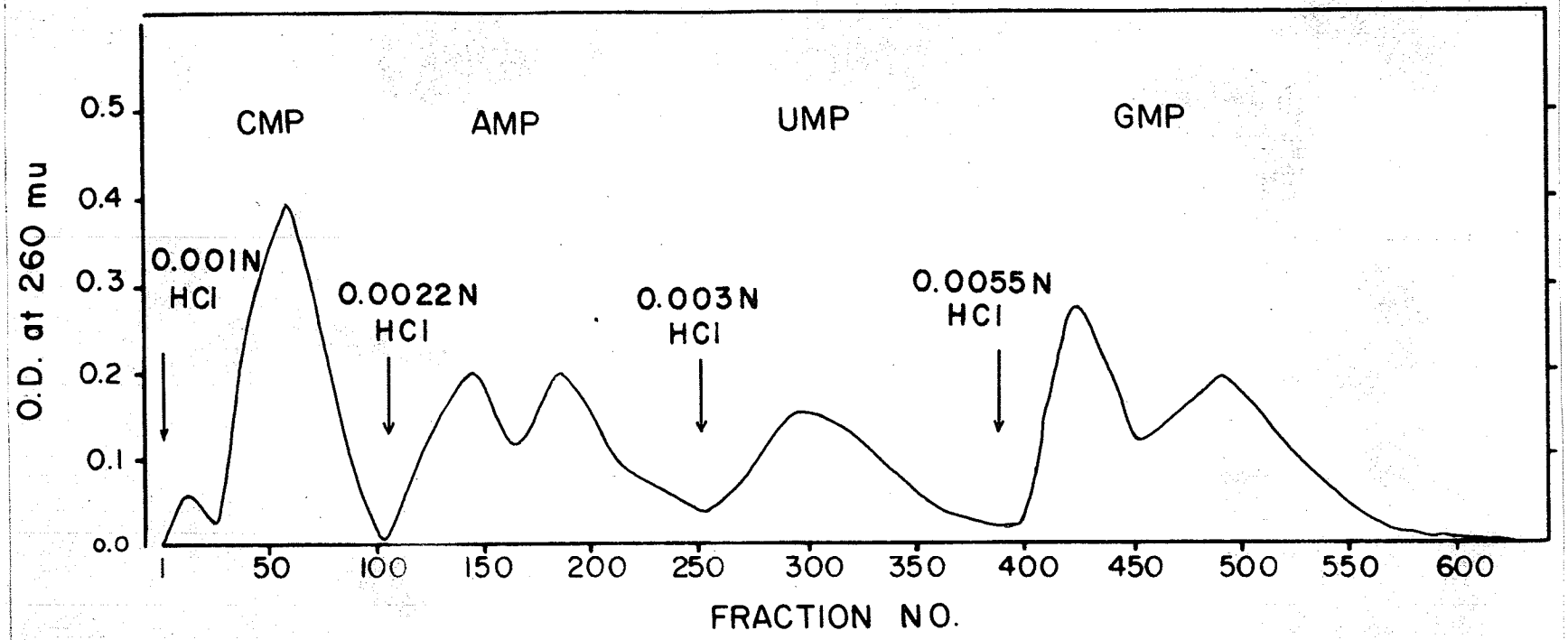
Chromatography on Dowex 1-Cl^- column for the separation of the nucleotides of the alkaline digest of sRNA was carried out with stepwise increases in HCl concentration to elute the nucleotides (see Fig. 3). Care was taken to adjust the pH of the hydrolysate to 4·0 and to remove excess K-perchlorate. As may be seen in Fig. 3, CMP was eluted from the column with 0·001 N HCl. AMP, which appeared as two peaks, was eluted with 0·0022 N HCl. Each of the two peaks has an absorption spectrum identical to that of AMP, presumably corresponding to the 2' and 3' mononucleotides. UMP was eluted at an HCl concentration of 0·003 N relatively slowly as indicated by spreading of the peak. GMP was eluted with 0·0055 N HCl as two peaks, presumably corresponding to the 2' and 3' mononucleotides. Fractions of each peak were pooled. The combined volume and its absorption spectrum were measured. The extinction coefficients (110) used in calculations for estimating the four nucleotides were as follows: CMP $E_{260} = 6{\cdot}8 \times 10^3$ (pH 2); AMP $E_{260} = 14{\cdot}2 \times 10^3$ (pH 2); UMP $E_{261} = 9{\cdot}7 \times 10^3$ (pH 2) and GMP $E_{260} = 11{\cdot}8 \times 10^3$ (pH 1).

The results of separation of nucleotides of sRNA alkaline hydrolysate were shown in Fig. 4 and Table 1. The pH of the sRNA hydrolysate was adjusted to 3·5 with 1 N PCA and excess of K-perchlorate was removed by centrifugation. The paper-strip

Fig. 3

Chromatographic pattern of

alkaline hydrolysate of rat liver sRNA



Total O.D. put on : 360 Units (at 260 mu)

Recovery of O.D. : 340 Units (at 260 mu), or 94 %

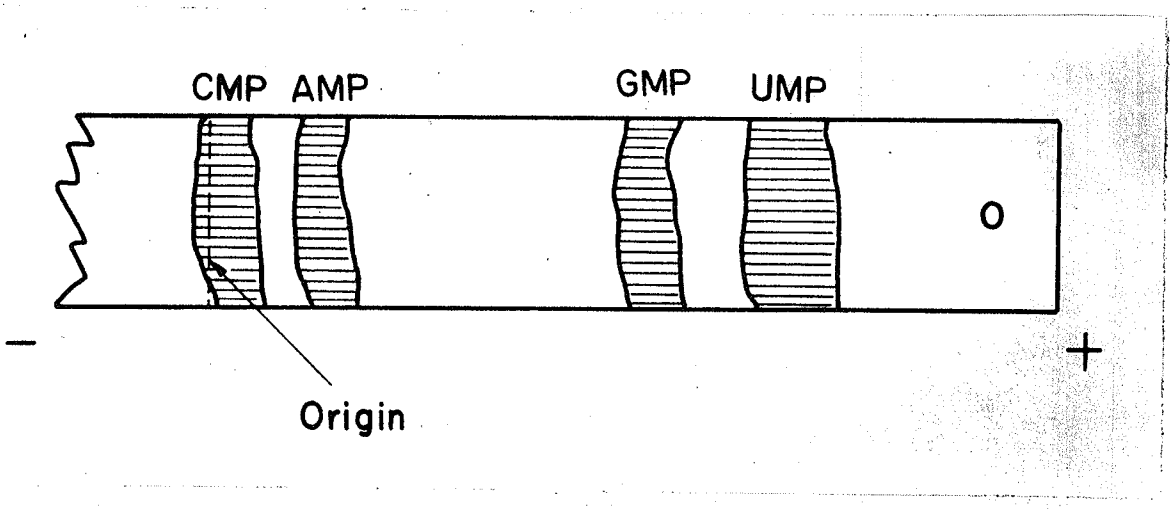
Column : Dowex 1-Cl^-, 1 x 8 cm

Flow rate : 0.5 ml/min at room temperature

Fraction volumn : 4.0 ml/tube

The Dowex column had been washed sufficiently with 0.1N HCl and finally it was washed with about 50 mls dist. water.

Fig. 4



Pattern of paper-strip electrophoresis
of alkaline hydrolysate of sRNA

Electrophoresis was carried out in 0.02M
Na-citrate buffer, pH 3.5, at room temperature
with a constant potential of 500 volts.

Table 1

Base Composition of Soluble RNA (moles %)

Source	AMP	GMP	CMP	UMP	Odd Bases
Rat liver sRNA (by Electrophoresis)	19·4 20·7 21·5	32·4 32·6 29·2	29·5 29·0 29·5	18·7 17·7 19·8	----
Rat liver sRNA (by chromatography)	19·9 21·5	31·5 31·5	29·4 29·7	19·2 17·3	----
Literature values					
Rat liver sRNA (116)‡	18·9	32·8	29·0	19·3	----
Guinea Pig Liver sRNA (117)	17·7	32·4	29·6	19·7	7·1
Mouse Ehrlich Ascites sRNA (118)	19·5	35·6	26·2	18·7	----
E. coli sRNA (115)	15·2	26·2	23·6	12·3	22·8
Baker's yeast sRNA (6)	18·9	34·6	28·5	18·0	----
Rat liver rRNA (116)	19·1	33·4	30·2	19·3	----
Mouse Ehrlich Ascites rRNA (118)	18·2	37·4	25·6	18·7	----

‡ Reference numbers are given in brackets.

electrophoresis was performed at room temperature in 0·02 M Na-citrate buffer at pH 3·5 as described in Materials and Methods.

Incorporation of 1-C^{14}-glycine into rat liver sRNA

The results obtained with the salt-ethanol washing procedure of Berg et al (Fig. 5, curve A) showed a higher specific activity than that obtained with a slightly modified TCA-washing procedure (Fig. 5, curve B). By calculation (based on the assumption that the M.Wt. for sRNA is 25,000) from the specific activities: it appeared that, in the material obtained with the salt-ethanol washing procedure (30), there is 2·1% of the unfractionated sRNA found to accept glycine compared to 1·65% in the material obtained by a modified TCA-washing procedure. The difference between the two procedures is thus about 22%.

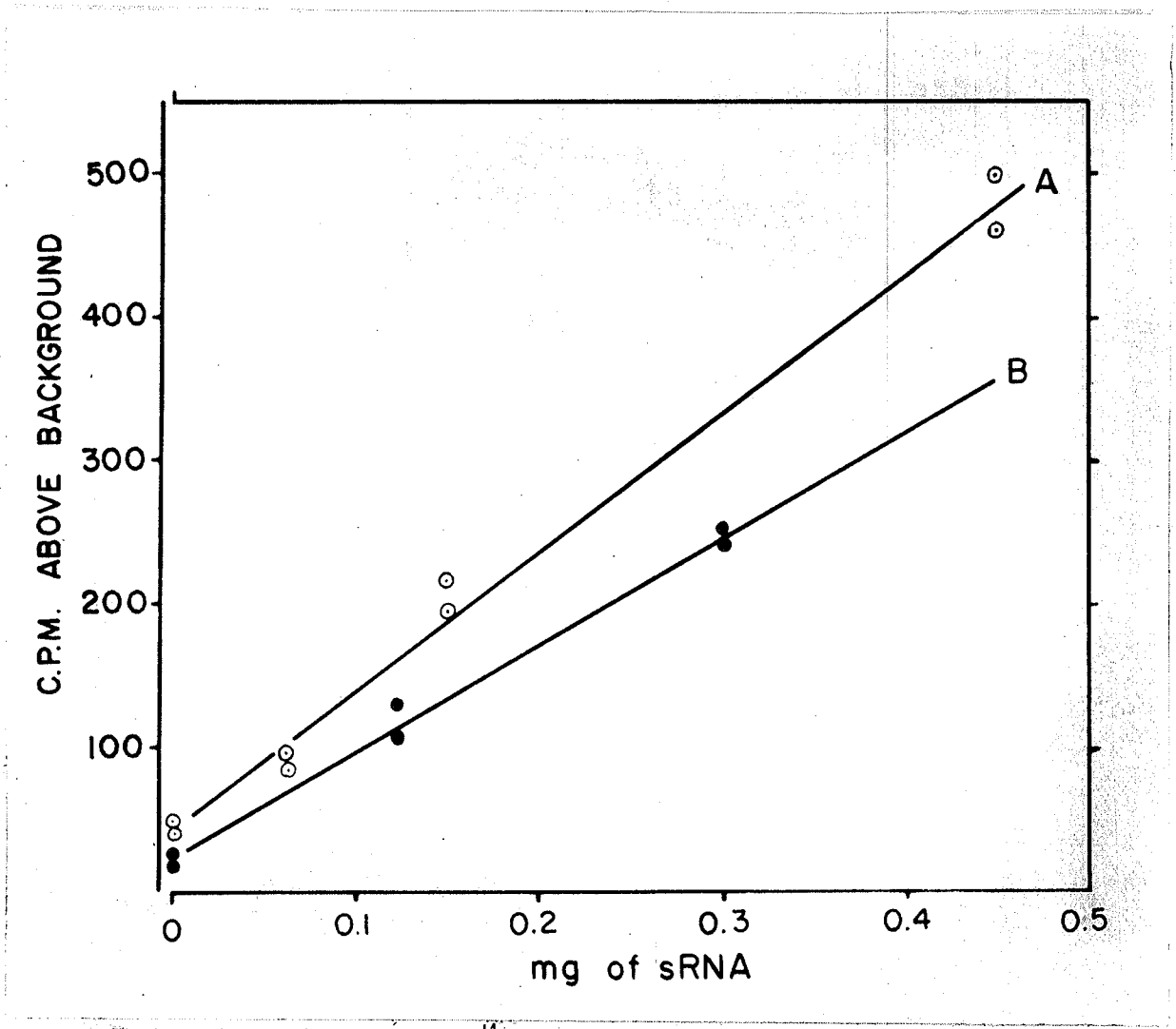
The incorporation of 1-C^{14}-glycine into sRNA was found to be proportional to the amount of sRNA present when the system was saturated with radioactive glycine and with excess of glycyl-RNA synthetase (50-60% $(NH_4)_2SO_4$ fraction).

Chromatography of sRNA on amino-ethyl (AE-50) cellulose column

a) Chromatography of free 1-C^{14}-glycine on AE-50 column

Preliminary chromatography of free standard 1-C^{14}-glycine on an AE-50 column was carried out with 0·005 M K-phosphate buffer at pH 8·2 as eluant. The procedure for the preparation of the column as described in Materials and Methods. The results of the chromatography are presented in Fig. 6. It may be seen that the radioactive glycine did not absorb on the column, but washed through in the first few fractions. About 83% of the radioactivity was

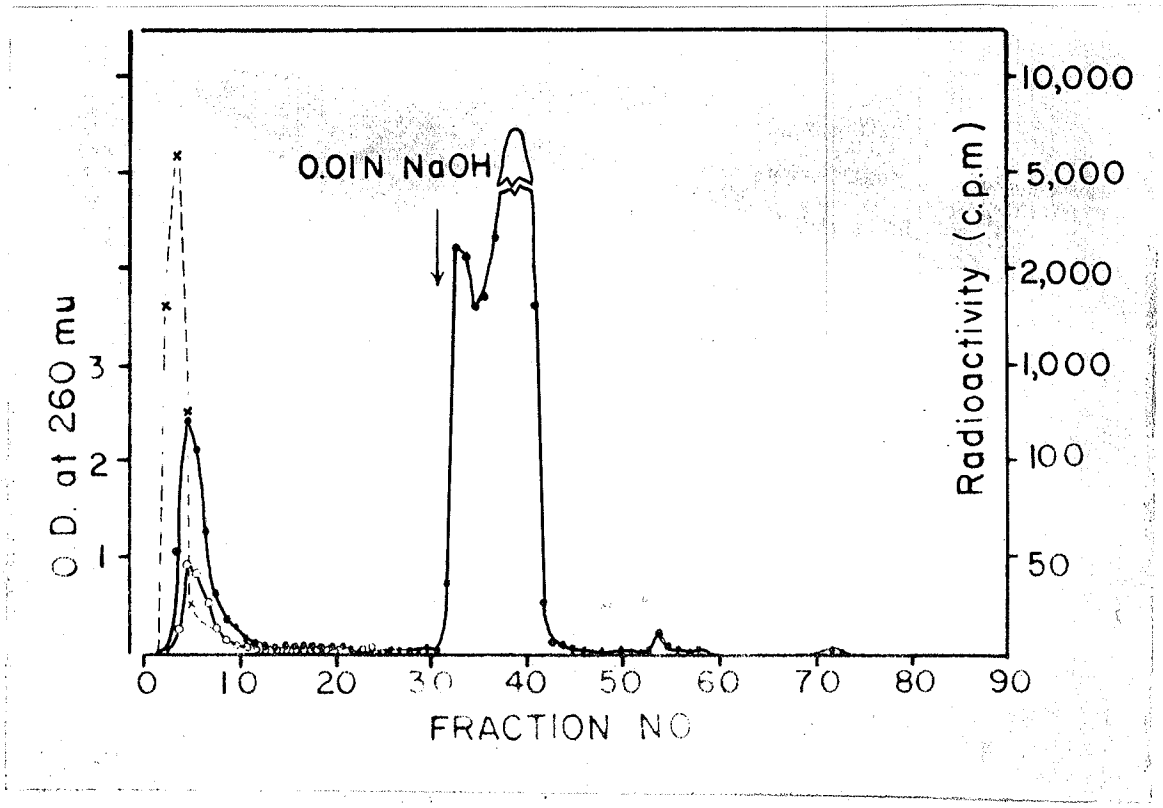
Fig. 5



Incorporation of 1-C^{14}-glycine into rat liver sRNA

The conditions for labeling of the sRNA were those described in Materials and Methods. Curve A was obtained by the salt-ethanol washing procedure of Berg et al (50). About 1.5 mg yeast RNA was added to each tube as carrier. Curve B was obtained by the TCA-washing procedure as described. Briefly, after the additions of 1.0 ml of 22.5% TCA with 2% glycine carrier and 1.0 ml"heated pH 5 enzymes" as protein-nucleic acid carrier the samples were washed twice with 7.5% TCA with 2% glycine carrier, once with 0.5M NaCl-67% ethanol mixture, twice with cold acetone and once with ether and finally each sample was suspended in 1.0 ml of ether and plated on a clean, lightly greased planchet and counted.
Separate blanks (no sRNA added) were run simultaneously for the two sets of samples.

Fig. 6



Chromatography of oxidized and 1-C^{14}-glycine-labeled sRNA on an AE-50 column with 0.005M K-phosphate buffer pH 8.2

Total O.D. put on : 210 O.D. units at 260 mu (10.5 mg).
Flow rate : 3 mls/min at room temperature.
Recovery of O.D. : 1 st peak, 13.3%
2 nd peak, 93%
Column height : 10 cm.

•—• represents O.D. at 260 mu.
o—o represents radioactivity of labeled sRNA.
×—× represents radioactivity of free standard 1-C^{14}-glycine (a separate experiment of chromatography of free standard 1-C^{14}-glycine under the identical conditions, see text).

Table 2

Glycine Acceptor Activities of Unfractionated and Fractionated sRNAs (see Fig. 6)

Samples	Amount of sRNA in mg	c.p.m.* obtained			specific activities μμmole/mg			Purification
		Before chromato-graphy	Calculated from the chromatogram	From relabelling experiment	Before chromato-graphy	Calculated from the chromatogram	From relabelling experiment	
Unfractionated sRNA	10·5	13,893	--	--	294	--	--	--
Fractionated sRNA Tubes 4-7 (Fig. 6)	1·399	--	5,280	2,569	--	838	408	$\frac{408}{294} = 1{\cdot}38$

Fractionated sRNA was obtained by the experiment shown in Fig. 6. sRNA in tubes 4-7 were recovered by dialysis overnight against two changes of distilled H_2O as described in Materials and Methods.

In relabelling experiment, 50-60% $(NH_4)_2SO_4$ fraction was used as the source of glycyl RNA synthetase in an incubation mixture described previously.

The specific activity of the fractionated sRNA as calculated from the chromatogram was not taken into account since, as indicated by the experiment of chromatography of free C^{14}-glycine under the same conditions, that the free C^{14}-glycine emerged in the same region as did the first peak of the sRNA fraction. It was not shown that all radioactivity present in these fractions was bound to sRNA.

*1 c.p.m. = 0·222 μμmoles of glycine.

recovered in the first 2 to 4 fractions. An identical pattern for free standard 1-C^{14}-glycine was obtained with 0·005 M K-phosphate buffer pH 6·0 as eluant also. The recovery of radioactivity in this case was 94% of that put on the column.

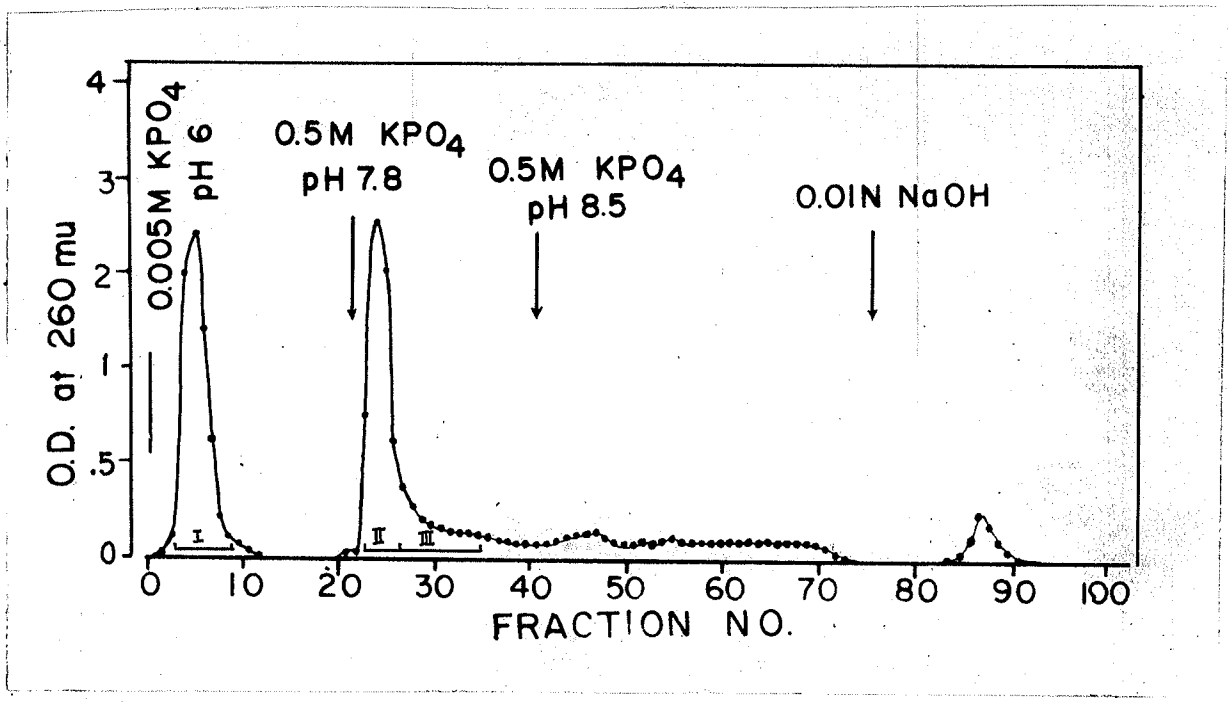
b) Chromatography of oxidized sRNA and 1-C^{14}-glycine labelled sRNA mixture on AE-50 column

A mixture of 1-C^{14}-glycine labelled sRNA and periodate oxidised sRNA was prepared according to the procedure described in Materials and Methods. The chromatography was carried out at room temperature with 0·005 M K-phosphate buffer pH 6·2 as eluant. The chromatographic profile is shown in Fig. 6. It was found that only one peak (III) was eluted with the buffer and no further sRNA was eluted until the column was treated with alkali. Only 13·3% of the U.V. absorbing material was recovered in the first peak (fractions 4 to 7). These fractions were pooled, dialysed overnight against two changes of distilled water and the volume of the dialysate was reduced by evaporation with a flash-evaporator (35-37°C). The ability of the fractionated sRNA to accept glycine was then determined. The specific glycine acceptor activity of the unfractionated material was 294 µmoles glycine/mg sRNA. The specific glycine acceptor activity of the fractionated sRNA was found to be 408 µmoles glycine/mg sRNA. This represents a purification of only 1·38-fold (see Table 2).

c) Chromatography of sRNA on AE-50 column with stepwise increases in concentration of K-phosphate

Phenol-extracted sRNA, free of bound amino acids, was

Fig. 7



Chromatography of stripped sRNA on an AE-50 column with stepwise eluting system of K-phosphate buffer at different pHs

Total O.D. put on : 83 O.D. units at 260 mu (4.15 mg).
Flow rate : 1.0 ml/min at room temperature.
Column height : 12.5 cm.
Recovery of O.D. : 1 st peak, 33.7%.
2 nd peak, 45%.

Glycine acceptor activities of pooled fractions (see Tab.3):
1 st peak, 123 moles glycine/mg sRNA (from a seperate experiment under the identical conditions).
2 nd peak, Tubes 23 - 26: 628 moles glycine/mg sRNA.
Tubes 27 - 36: 1,330 moles glycine/mg sRNA.

Table 3

Glycine Acceptor Activity of Fractionated sRNA

sRNA Sample	Tube No.	Amount of sRNA (mg)	c.p.m.* obtained	c.p.m. corrected for blanks	Specific Activity µmoles/mg
No sRNA	1 2	--	124·0 124·4	--	--
Stripped sRNA	3 4	0·095 0·095	232·4 214·2	99·1	946
Fractionated sRNA (†Fraction II)	5 6	0·092 0·092	185·7 189·6	63·4	628
Fractionated sRNA (†Fraction III)	7 8	0·022 0·022	162·6 149·8	32	1330

The specific activity of sRNA in Fraction I as determined from a separate experiment under the identical condition was 123 µmoles glycine/mg sRNA.

†Fractions were obtained from the experiment as shown in Fig. 7. These fractions were recovered by dialysis against 2 changes of distilled water, evaporated by flash evaporator as described.

The conditions for labelling were the same as described. Samples were washed according to the procedure of Berg et al. 50-60% $(NH_4)_2SO_4$ fraction was used as glycyl RNA synthetase source.

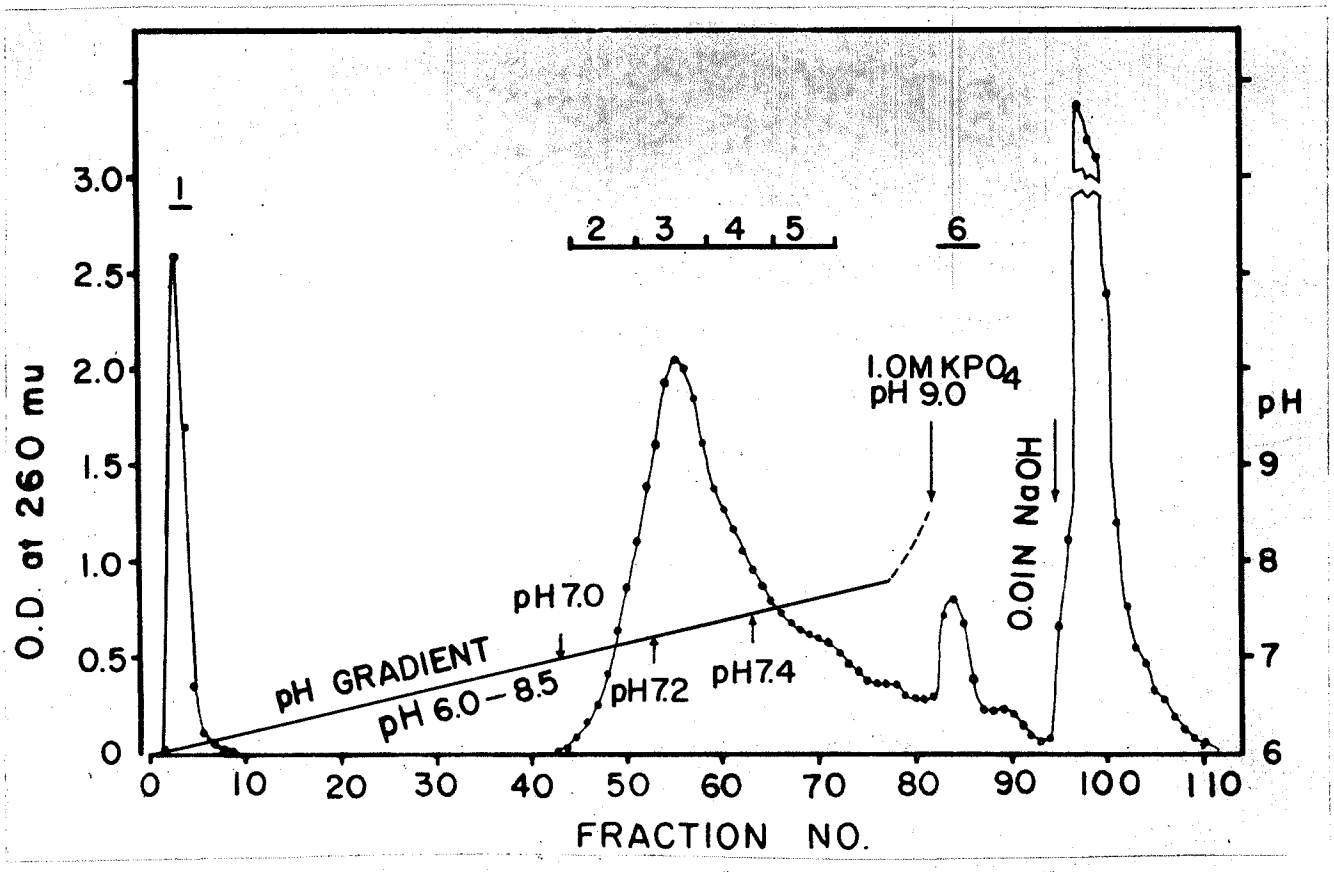
*1 c.p.m. = 0·91 µmoles of C^{14}-glycine.

fractionated by chromatography on AE-50 column at room temperature by elution with stepwise increases in concentration and/or pH of the phosphate buffer. The chromatographic profile may be seen in Fig. 7. With 0·005 M K-phosphate buffer, pH 6·0, about 34% of the U.V. absorbing material was recovered. When the eluent was changed to 0·5 M K-phosphate buffer, pH 7·6, a second peak, containing 45% U.V. absorbing material, was eluted. With 0·5 M pH 8·5 K-phosphate buffer, a further 14% of the U.V. absorbing material was eluted. Tests of the glycine acceptor activity of the sRNA eluted in these peaks were carried out as described previously. The results are presented in Table 3. The specific activity of the first peak was found to be only 123 μμmoles glycine/mg sRNA (as determined from a separate experiment) while the specific activity in the second peak varied. Fractions which come through the column earlier had a specific activity of 628 μμmoles glycine/mg sRNA whereas the later fractions had a specific activity of 1330 μμmoles glycine/mg sRNA (see Table 3).

d) Chromatography of sRNA on AE-50 column with a near linear pH gradient eluting system.

Attempts were also made to fractionate the sRNA by chromatography on AE-50 column with a near linear pH gradient (pH 6-8·5) of 0·5 M K-phosphate buffer. In this experiment phenol-extracted sRNA was used and chromatography was done at room temperature. It may be seen in Fig. 8 that the first peak, containing about 6·5% of the U.V. absorbing material, came through in the first few fractions and the second peak started to come through from the column when the pH rose to about 7·0. At the end of the

Fig. 8



Chromatography of intact sRNA (phenol-extracted) on an AE-50 column with pH gradient eluting system of 0.5M K-phosphate buffer (pH 6.0 - 8.5)

Total O.D. put on : 300 O.D. at 260 mu (15 mg).
Flow rate : 0.33 mls/min at room temperature.
Column height : 14.5 cm.
Recovery of O.D. : Tubes 3 - 5, 6.5%.
Tubes 42 - 94, 52.5%.

Glycine acceptor activities of pooled fractions: (Tab. 4)

Fr. 1,	0.0	moles glycine/mg sRNA.
Fr. 2,	1,155	moles glycine/mg sRNA.
Fr. 3,	2,460	moles glycine/mg sRNA.
Fr. 4,	965	moles glycine/mg sRNA.
Fr. 5,	1,693	moles glycine/mg sRNA.
Fr. 6,	1,765	moles glycine/mg sRNA.

During the run the pH of certain fractions were measured in order to ascertain the pH of the fractions collected and the results of these measurements indicated that the pH gradient appeared to be near linear.

Table 4

Glycine Acceptor Activities of Fractionated sRNA Obtained from Fig. 8.

sRNA Sample	Tube No.	Amount of sRNA (mg)	c.p.m. obtained	c.p.m. corrected for blanks	Specific Activity μμmoles/mg glycine	Purification
No sRNA	1 2	--	144·4 145·0	--	--	--
Unfractionated sRNA	3 4	0·114	271·9 261·1	121·8	965	--
Fraction 1	5 6	0·114	142·2 134·7	0	0	0
Fraction 2	7 8	0·087	263·1 247·8	110·8	1155	1·2
Fraction 3	9 10	0·117	466·2 453·8	315·3	2460	2·6
Fraction 4	11 12	0·102	247·5 258·1	108·1	965	1
Fraction 5	13 14	0·039 0·078	219·4 286·6	74·7 141·9	1730 1655	1·8
Fraction 6	15 16	0·112	373·7 351·6	217·9	1765	1·8

*1 c.p.m. = 0·91 μμmoles of glycine.

In this experiment 50-60% $(NH_4)_2SO_4$ fraction was used as the source of glycyl RNA synthetase.

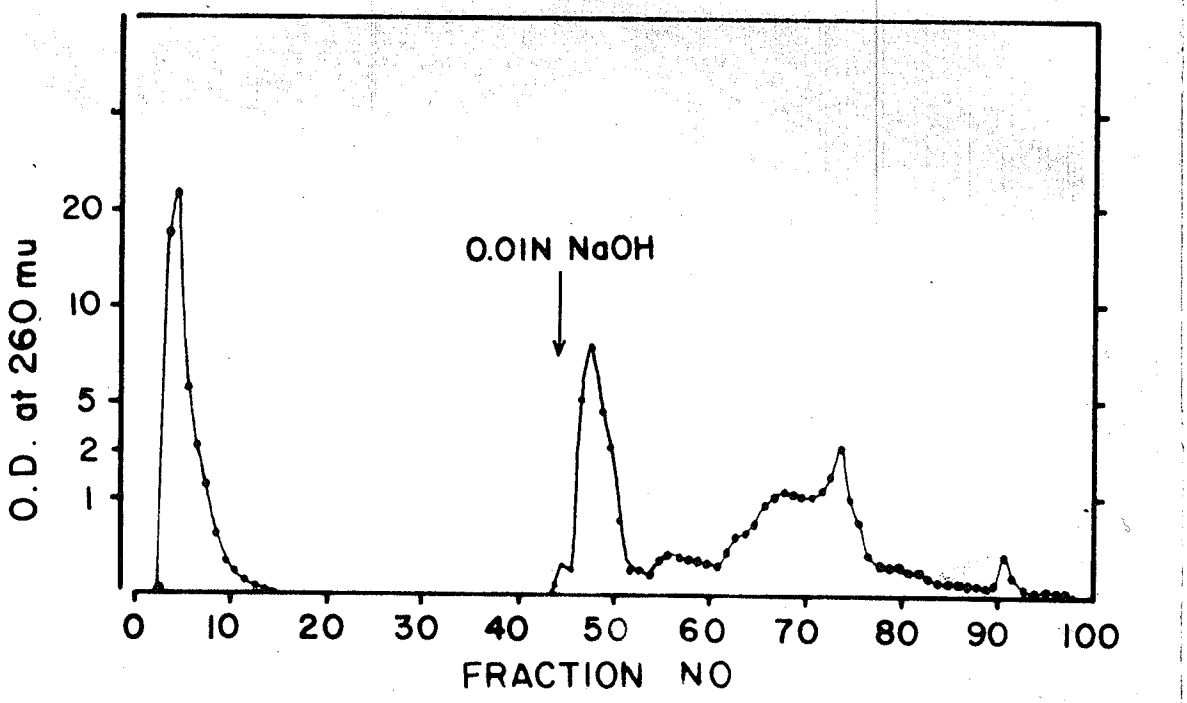
Conditions for assaying the acceptor activity of sRNA were the same as described previously. The samples were washed by the method of Berg et al.

gradient the elution was continued with 1·0 M K-phosphate buffer, pH 9·0, so that a third peak was obtained. Nevertheless, about 40% of the U.V. absorbing material still remained on the column. This was eluted with alkali (see Fig. 8). Fractions in the second peak of the chromatogram were subsequently combined into four pooled fractions. These four fractions as well as those in the first and third peaks were all dialysed over-night against distilled water to remove the buffer salts. The sRNAs were then recovered as described previously and their glycine acceptor activities determined. The results are presented in Table 4. It may be seen that peak 1 had no measureable glycine acceptor activity, while different regions of the second peak had different glycine acceptor activities. A fraction of sRNA with a specific activity of 2,460 µmoles glycine/mg sRNA was obtained from elution of pH 7·15-7·3. This represents a purification of 2·6-fold and a purity of 6·2% (calculated by assuming that the M.Wt. of sRNA is 25,000 and 1 mole of glycine is bound per mole of glycyl RNA).

e) Chromatography of sRNA on AE-50 column with an eluting system of 3·0 M Na-formate and 0·5 volume% of triethanolamine pH 8·2

Phenol-extracted sRNA was fractionated by chromatography on AE-50 column with an eluting buffer (45) containing 3·0 M Na-formate and 0·5 volume% triethanolamine, pH 8·2 (see Fig. 9). Only about 61% of the U.V. absorbing material was eluted from the column at room temperature. The remainder was eluted with alkali. Under identical conditions the chromatographic pattern of the periodate oxidized sRNA was found to be essentially the same as that

Fig. 9



Chromatography of intact sRNA on an AE-50 column with 3.0M Na-formate and 0.5 volume% triethanolamine at pH 8.2

Total O.D. put on : 492 O.D. units at 260 mu (24.6 mg).
Flow rate : 0.5 mls/min at room temperature.
Column height : 12 cm.
Recovery of O.D. : 1 st peak, 61%.
2 nd peak, 40%. (eluted with alkali).

A separate experiment (referred to in the text) of chromatography of oxidized sRNA under the same conditions was found to be essentially the same as above.
Total O.D. put on : 314 O.D. units at 260 mu (15.7 mg).
Flow rate : 0.6 mls/min at room temperature.
Column height : 8 cm.
Recovery of O.D. : 1 st peak, 23.5%
2 nd peak, -

Note: intact sRNA referred to as phenol-extracted sRNA in the text.

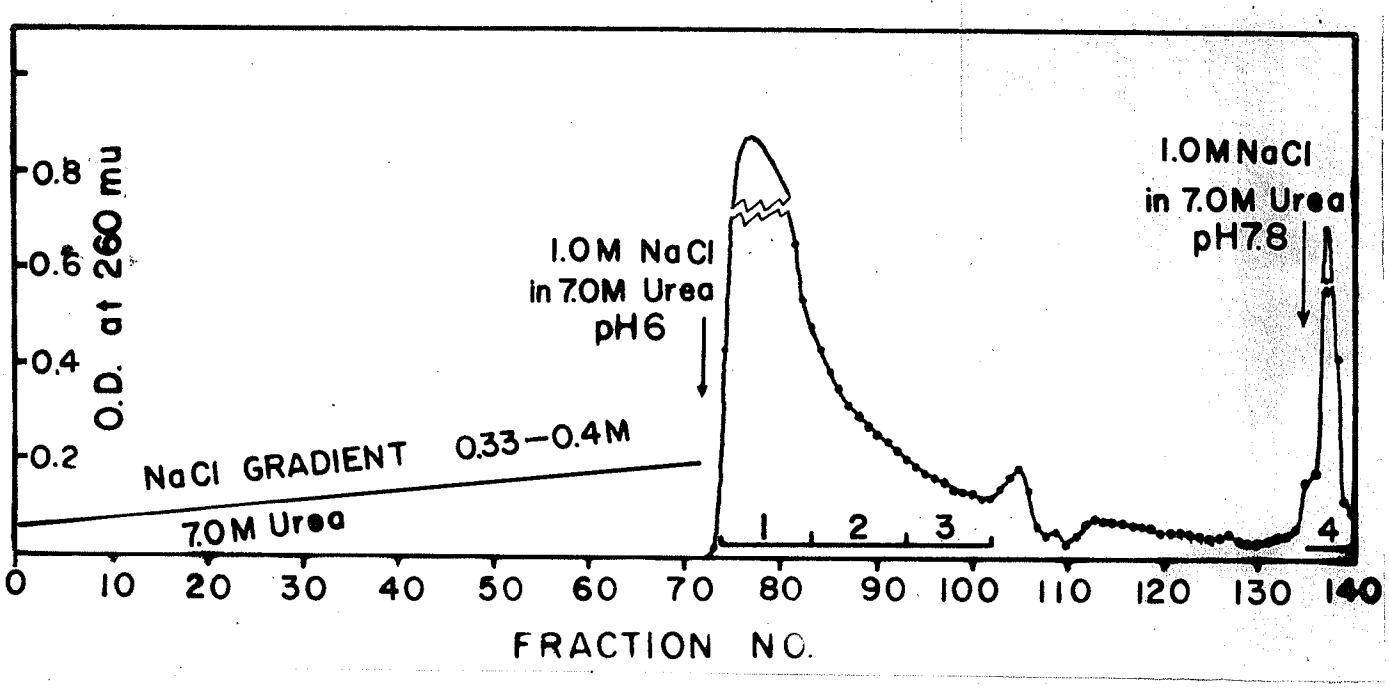
obtained with intact sRNA (see Fig. 9) but only differ in that the recovery of the U.V. absorbing material in the buffered eluate was about 23·5% of the total compared to 60% for the intact sRNA. A separate experiment was carried out in which periodate oxidized sRNA was chromatographed in the same buffer system, but with a longer column (50 cm) and at a lower temperature (0^{o}-4^{o}C). A chromatographic profile similar to that shown in Fig. 9 was obtained. The recovery in the first peak was 22·8%.

When the oxidized and 1-C^{14}-glycine-labelled sRNA had been kept in the column at room temperature for one hour prior to starting the elution with the same buffer system as just mentioned, only slightly different results were obtained (see Fig. 10). Under these conditions the first peak was found to contain 18% of the sRNA that was put on the column.

f) Chromatography of sRNA in a NaCl linear gradient in 7·0 M urea and 0·1 M K-phosphate buffer, pH 6·0.

A preliminary chromatographic experiment (see Fig. 11) showed that purified sRNA did not elute from an AE-30 column in a NaCl linear gradient (0·33-0·4 M) in the presence of 7·0 M urea (113) and 0·1 M K-phosphate buffer pH 6·0. When the concentration of NaCl was raised to 1·0 M in the presence of 7·0 M urea and 0·1 M K-phosphate buffer pH 6·0 a major portion of sRNA was eluted. Based on these results an eluting system was adopted which employed a NaCl linear gradient (0·4-1·5 M) in the presence of 7·0 M urea and 0·1 M K-phosphate buffer, pH 6·0, at room temperature (see Fig. 12). As observed in the previous experiment, no U.V. absorbing material was found in the fractions of low salt concentration. The bulk of the sRNA eluted from the column in the concentration range

Fig. 11



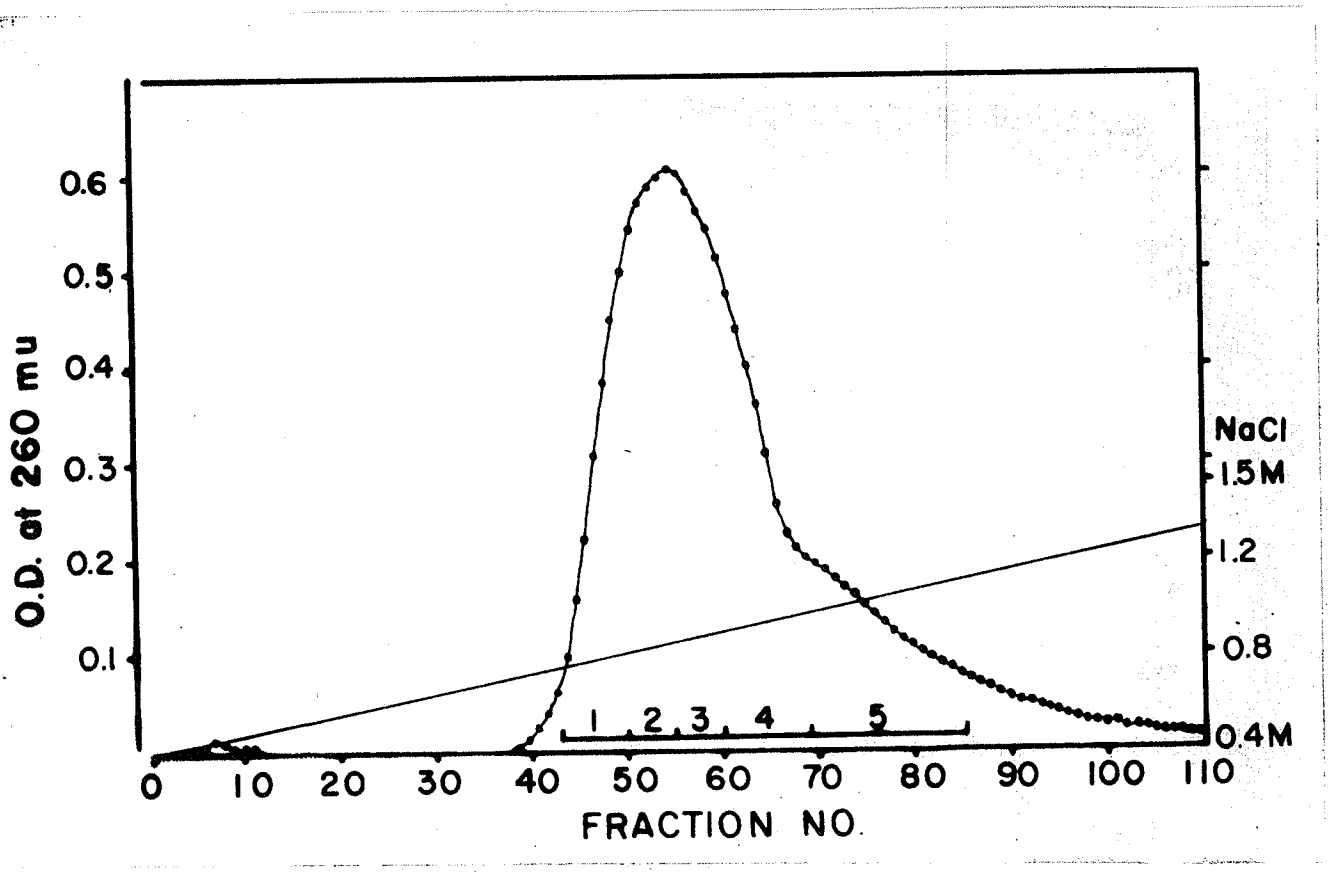
Chromatography of purified sRNA on an AE-50 column with NaCl linear gradient in 7.0M urea and 0.1M K-phosphate buffer at pH 6.0

Since the sRNA did not elute with an eluting system of 0.33 - 0.4M NaCl linear gradient in 7.0M urea and 0.1M K-phosphate at pH 6.0 so that elutions of 1.0M NaCl in 7.0M urea and 0.1M K-phosphate buffer at pH 6.0 and at pH 7.0 were used.

Total O.D. put on : 208 O.D. units at 260 mμ (10.4 mg).
Flow rate : 2 mls/min at room temperature
Column height : 16 cm
Recovery of O.D. : Tubes 75 - 134, 82%
Tubes 135 - 142, 4.8%

Glycine acceptor activities of pooled fractions:
Fr. 1, 1,490 moles glycine/mg sRNA; Fr. 2, 1,270 moles glycine/mg sRNA; Fr. 3, 482 moles glycine/mg sRNA; Fr. 4, 1,300 moles glycine/mg sRNA.

Fig. 12

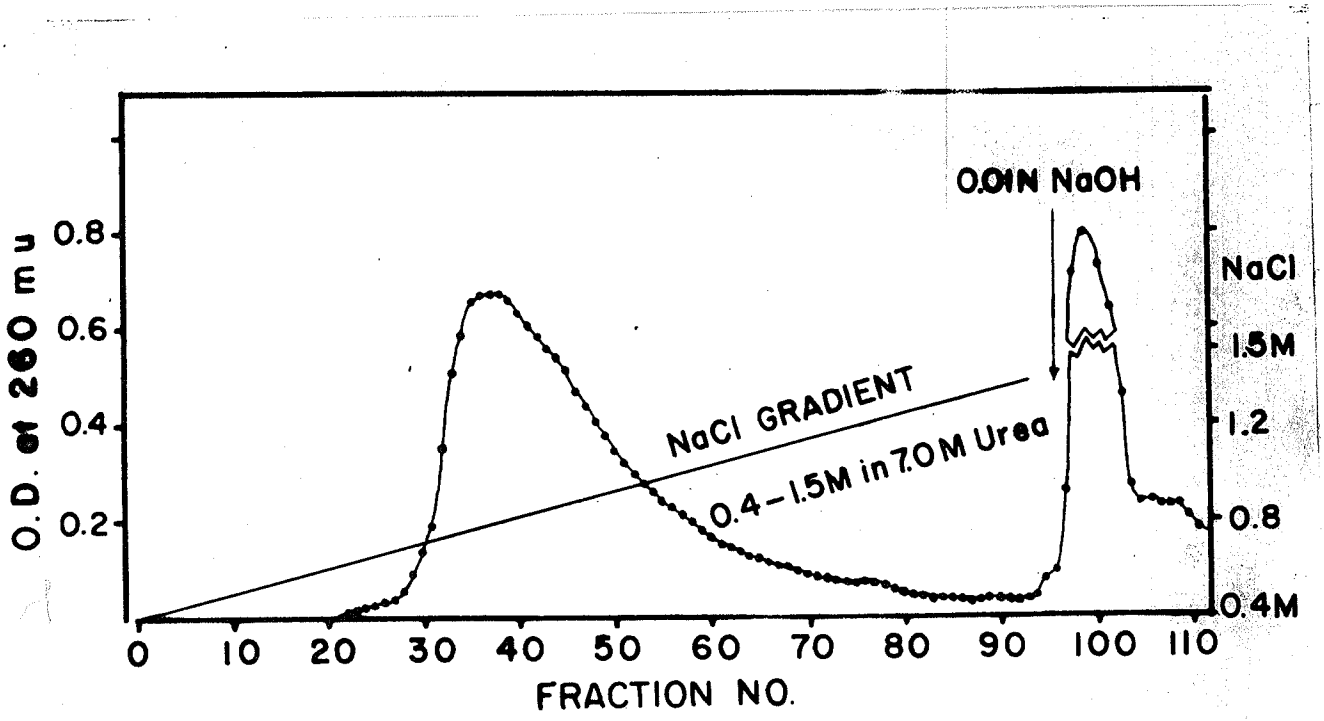


Chromatography of purified sRNA on an AE-50 column with 0.4 - 1.5M NaCl linear gradient in 7.0M urea and 0.1M K-phosphate buffer at pH 6.0

Total O.D. put on : 130 O.D. units (6.5 mg sRNA)
Flow rate : 1.0 ml/min at room temperature
Column height : 16 cm
Recovery of O.D. : 95 - 96%
Glycine acceptor activities of pooled fractions :

Fr. 1, 1,400 moles glycine/mg sRNA
Fr. 2, 1,860 moles glycine/mg sRNA
Fr. 3, 1,870 moles glycine/mg sRNA
Fr. 4, 1,870 moles glycine/mg sRNA
Fr. 5, 1,980 moles glycine/mg sRNA

Fig. 13



Chromatography of oxidized sRNA on an AE-50 column with 0.4 - 1.5M NaCl linear gradient in 7.0M urea and 0.1M K-phosphate buffer at pH 6.0

Total O.D. put on : 107 O.D. units at 260 mu (5.35 mg).
Flow rate : 2 mls/min at room temperature.
Column height : 16 cm.
Recovery of O.D. : 1 st peak, 64%
2 nd peak, 34.7% (eluted with alkali)

Glycine acceptor activity of the pooled fraction from the first peak : 22.1 moles glycine/mg sRNA (see Table 5).

Table 5

Glycine Acceptor Activity of Oxidized sRNA Fraction from Fig. 13.

sRNA Sample	Tube No.	c.p.m.* obtained	c.p.m. corrected for blanks	Specific Activity µµmoles/mg glycine
No sRNA	1 2	113·7 103	--	--
Unfractionated sRNA 0·076 mg/tube	3 4	217·2 231·4	115·9	1380
Oxidized sRNA‡ 0·144 mg/tube	5 6	117·5 106·3	3·5	22·1

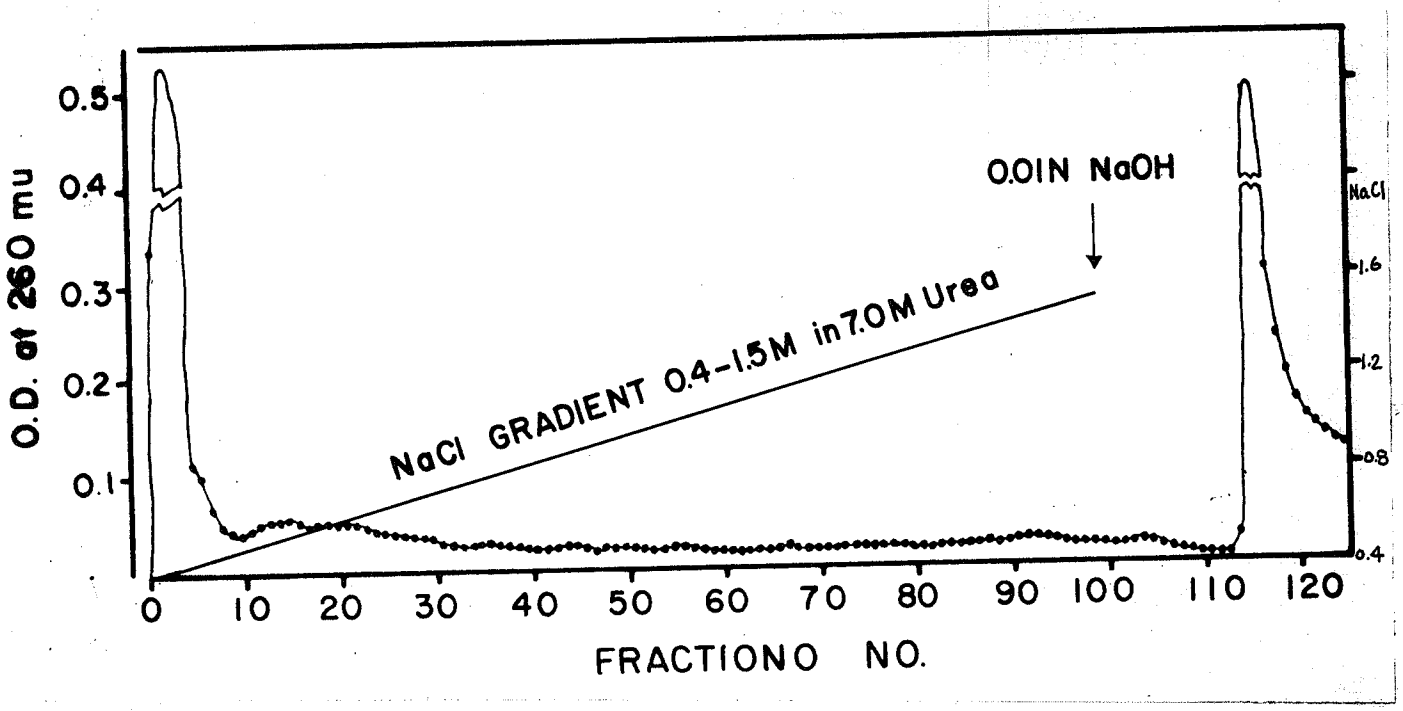
In this experiment 50-60% $(NH_4)_2SO_4$ fraction was used as the source of glycyl RNA synthetase.

Conditions for labelling were the same as described in Materials and Methods. The samples were washed according to the procedure of Berg et al.

‡obtained from the first peak of Fig. 13 and was recovered by dialysis as described elsewhere.

*1 c.p.m. = 0·91 µµmoles of glycine.

Fig. 14



Chromatography of oxidized sRNA on an AE-50 column with 0.4 - 1.5M NaCl linear gradient in 7.0M urea and 0.1M K-phosphate buffer at pH 8.2

Total O.D. put on : 81 O.D. units at 260 mu (4.05 mg).
Flow rate : 1.2 mls/min at 0 - 4 C
Column height : 50 cm.
Recovery of O.D. : 1 st peak, 50.6%
2 nd peak, 44.8% (eluted with alkali).

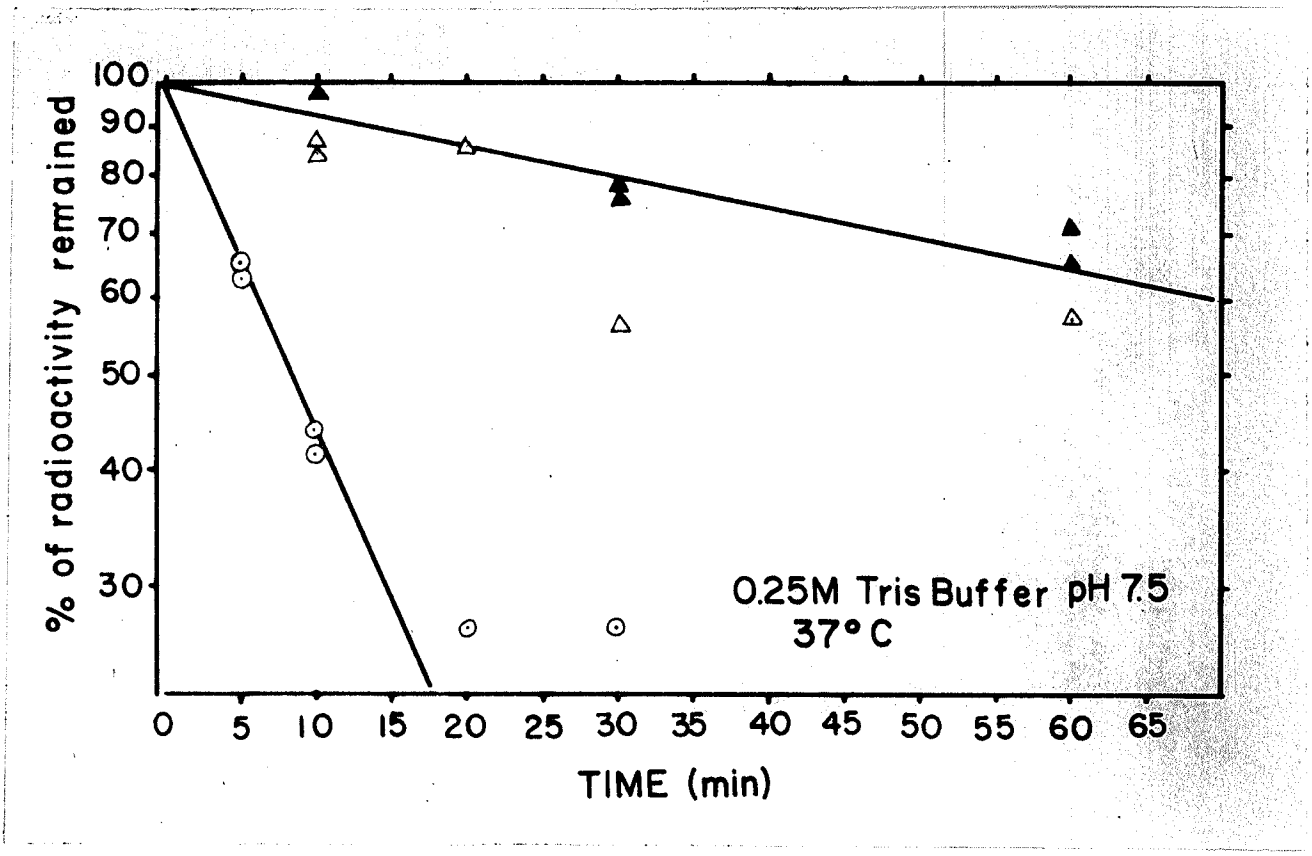
0·7-1·4 M NaCl as a single peak. The recovery of the U.V. absorbing material was 96%. Various fractions in this peak were pooled and the sRNA in these combined fractions was recovered as described previously. The glycine acceptor activities of the combined fractions were then tested. The results showed that there was no appreciable difference in specific glycine acceptor activity of the various fractions in the peak (see Fig. 12).

Periodate oxidized sRNA was also fractionated by column chromatography with the same NaCl gradient eluting system at room temperature. The results (see Fig. 13) show that the oxidized sRNA emerges from the same region as that of untreated sRNA, although the amount of U.V. absorbing material recovered in the peak was only 64% as compared to 96% recovery in the last experiment (Fig. 12). It may be seen from the data presented in Table 5 that the oxidized sRNA recovered from the first peak showed no significant glycine acceptor activity.

In a separate experiment, chromatography of oxidized sRNA using the same eluting system was performed in the cold with a longer column (52 cm). The chromatographic profile was found to be essentially the same as that described in Fig. 13, but only 45% of the oxidized sRNA was recovered in the first peak.

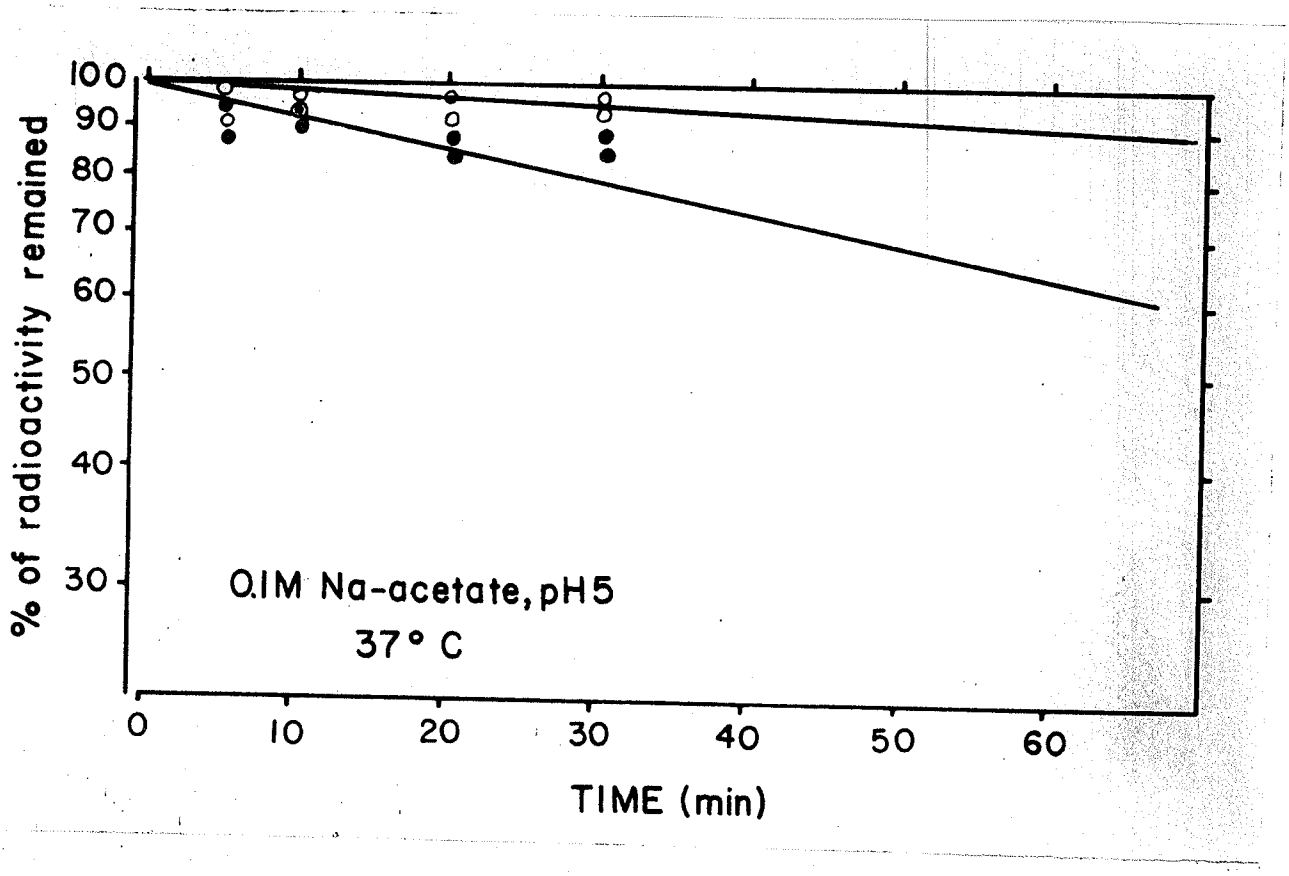
If the pH of the NaCl gradient eluting system was changed from 6·0 to 8·2 and the chromatography was also performed in the cold (see Fig. 14), then the oxidized sRNA was eluted much earlier

Fig. 15



Stability of acetylated 1-C^{14}-glycine-labeled sRNA
Incubation were carried out in 0.25M Tris-HCl buffer, pH 7.5, at 37°C.
⊙ Unacetylated 1-C^{14}-glycine-labeled sRNA.
△ Acetylated 1-C^{14}-glycine-labeled sRNA.(▲from separate expt.)
The ordinate scale is logarithmic.

Fig. 16



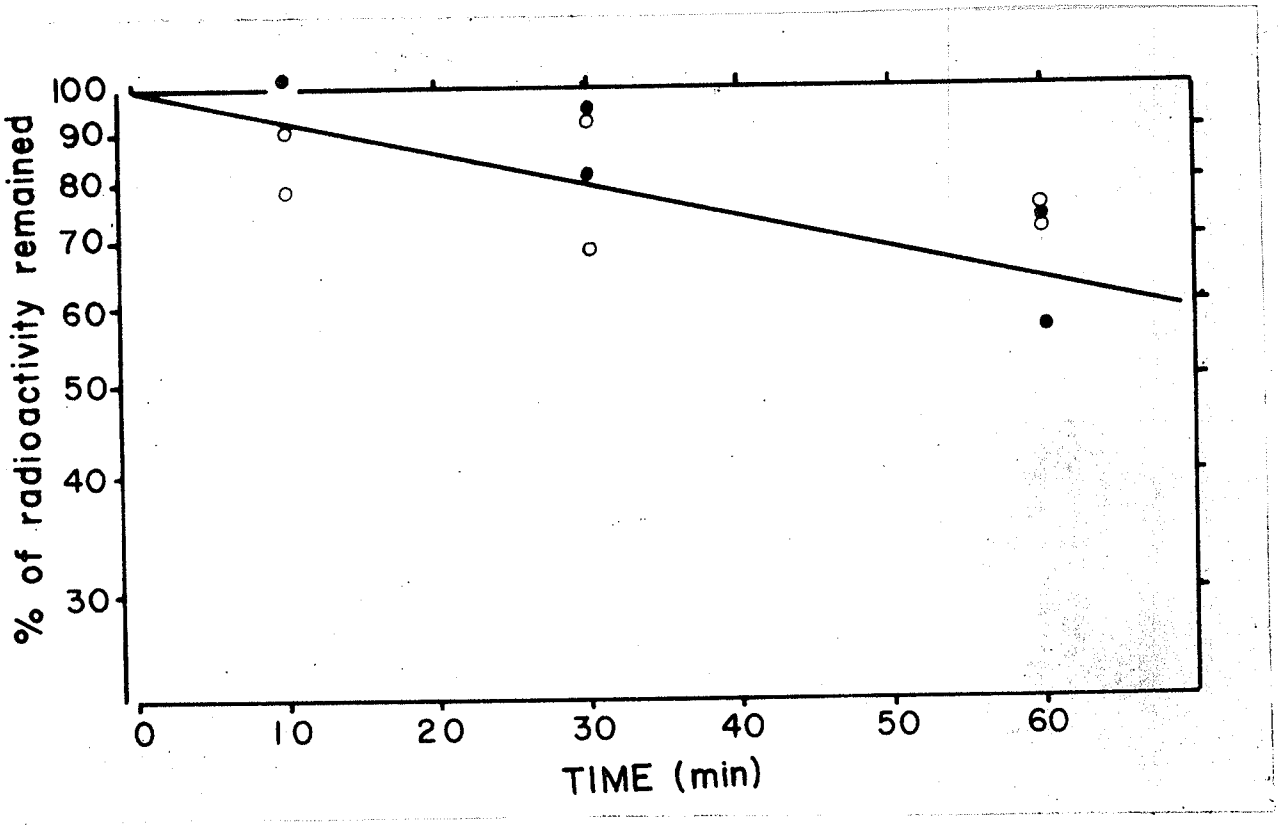
Stabilities of 1-C^{14}-glycine-labeled sRNA and of acetylated 1-C^{14}-glycine-labeled sRNA at pH 5.0 Incubation were carried out in o.1M Na-acetate buffer, pH 5.0, at 37°C.

• Unacetylated 1-C^{14}-glycine-labeled sRNA.

○ Acetylated 1-C^{14}-glycine-labeled sRNA.

The ordinate scale is logarithmic.

Fig. 17



Effect of acetylation on the glycine acceptor activity of sRNA (see also Table 6)
Incubation were carried out in 0.25M Tris-HCl buffer, pH 7.5, at 37°C.
● Control sample, acetylated after labeling.
○ sRNA acetylated before and after labeling.
The ordinate scale is logarithmic.

Table 6

Effect of Acetylation on Glycine Acceptor Activity of Rat Liver sRNA

Sequence of Treatments	sRNA (A)* (11·6 mg)	sRNA (B) (8·8 mg)
Incubation at pH 10 for 30 min. at 37°C	+	+
Acetylation	+ After acetylation the A 280/260 = 0·532	− No acetylation, the A 280/260 = 0·474
Labelling with 1-C^{14}-glycine	+	+
Acetylation of C^{14}-glycine labelled sRNA	+	+
	After acetylation and well washed with ethenol both sample were each dissolved up in 4·0 ml of H_2O. 0·1 ml portions were removed from each sample for O.D. and radioactivity measurements	
	100λ gave 106·4 c.p.m. 100λ gave 111·5 c.p.m. 100λ + H_2O → 10·0 ml A 260 = 0·79 A 280 = 0·222 A $\frac{280}{260}$ = 0·28	100λ gave 117·8 c.p.m. 100λ gave 132·1 c.p.m. 100λ + H_2O → 10·0 ml A 260 = 0·78 A 280 = 0·216 A $\frac{280}{260}$ = 0·277

*Sample A and B were run parallelly in the labelling experiment and also in the acetylation treatment.

+ come through the treatment.

− no treatment.

eluting from the column at the starting concentration of 0·4 M NaCl. The recovery of oxidised sRNA in the first peak was 50·6%.

Acetylation of 1-C^{14}-glycine-labelled sRNA

The results of experiments to test the stability of acetylated 1-C^{14}-glycine-labelled sRNA in 0·25 M Tris-HCl buffer at pH 7·5 at 37°C are shown in Fig. 15. It may be seen that the half-life for acetylated 1-C^{14}-glycine-labelled sRNA was found to be about 1·5 hours compared to the half-life of 8-9 minutes for the untreated 1-C^{14}-glycine-labelled sRNA (61) under the same conditions. In 0·1 M Na-acetate pH 5·0 at 37°C (see Fig. 16) the half-life for acetylated 1-C^{14}-glycine-labelled sRNA was found (extrapolated) to be longer than 6 hours. The half-life for untreated 1-C^{14}-glycine-labelled sRNA, under these conditions, was only 1·5 hours.

That acetylation does not inactivate or destroy the glycine acceptor activity is indicated by the following experiment (see Fig. 17). Phenol-extracted sRNA, free of bound amino acids, was put through the acetylation procedure before it was labelled with 1-C^{14}-glycine. This sRNA was found to accept radioactive glycine to the same extent as did the control sample (see Table 6), and its stability curve in 0·25 M Tris-HCl buffer pH 7·5 at 37°C followed the same course as that of the control sample (Fig. 17).

Reconstitution of pancreatic RNase-treated sRNA

As shown in Table 7, the glycine acceptor activity of the pancreatic RNase-treated sRNA was restored on the addition of nucleoside triphosphates when incubated with the "pH 5 enzymes"

Table 7

Reconstitution of Pancreatic RNase-treated sRNA

Incubation Mixture Plus	Amount of sRNA (mg)	Incorporation of C^{14}-glycine into sRNA		
		c.p.m. obtained	Specific Activity c.p.m./mg	% of reconstitution
- sRNA	--	101 97·8	--	--
+ untreated sRNA	0·103	128 116	220	--
+ control sRNA	0·258	178 162	270	--
+ treated sRNA (0·0001 µg RNase/ml)*	0·255	141 145	171	67
+ treated sRNA + CTP	0·255	154·2 156·2	219	86
+ treated sRNA + CTP + UTP + GTP	0·255	165·8 173·4	275	108
- sRNA	--	60·2 64·5	--	--
+ untreated sRNA	0·159	67·8 74·2	56·2	--
+ control sRNA	0·376	70·8 81·4	36·4	--
+ treated sRNA (0·0001 µg RNase/ml)**	0·339	61·2 64·5	1·5	3·5
+ treated sRNA + CTP	0·339	67·6 64·1	10·8	25
+ treated sRNA + CTP + UTP + GTP	0·339	76·8 69·4	33	76
+ treated sRNA (0·0001 µg RNase/ml)*	0·37	67·8 69·0	16·2	37
+ treated sRNA + CTP	0·37	70·1 73·6	25·6	59
+ treated sRNA + CTP + UTP + GTP	0·37	80·3 80·2	48	110
+ treated sRNA (0·001 µg RNase/ml)*	0·376	64·4 65·2	6·4	14·7
+ treated sRNA + CTP	0·376	64·0 70·8	3·3	30·5
+ treated sRNA + CTP + UTP + GTP	0·376	75·2 82·7	44	101

See separate page for legend to table.

Legend for Table 7

Incubation mixture contained 10 μmoles $MgCl_2$, 10 μmoles mercaptoethanol, 50 μmoles Tris-HCl buffer pH 7·5, 5 μmoles ATP pH 7·5 and with or without added sRNA and other nucleoside triphosphates (final concentration 1 μmole per ml) as specified. The mixtures were pre-incubated at 37°C for 20 minutes with 0·1 ml pH 5 enzyme in a final volume of 0·95 ml. Then 0·05 ml 12·9 mM C^{14}-glycine (final concentration 0·65 μmole/ml) was added to each tube and the mixtures were incubated for an additional 10 minutes, after which 1·5-2·0 mg yeast RNA was added to each incubation and followed quickly by the addition of 3·0 volume of 0·5 M NaCl 67% ethanol as described previously.

The average specific activities of untreated, control and RNase treated (in the presence of four nucleoside triphosphates) sRNAs were taken as 100% acceptor activity for the glycyl RNA.

Untreated sRNA: same as phenol-extracted sRNA, prepared by the phenol method of Kirby, see Materials and Methods.

Control sRNA: phenol-extracted sRNA which had been briefly shaken with phenol before the addition of RNase, see p.30.

*Aged RNase, $Fresh RNase, see p.42.

Table 8

Reconstitution of Pyrophosphorlyzed sRNA

Incubation Mixture Plus	C.P.M. INCORPORATION							
	No preincubation		Preincubation, at 37°C for 20 minutes					
	mg sRNA	Expt. 1 (30' pp-lysis)	mg sRNA	Expt. 2 (30' pp-lysis)	mg sRNA	Expt. 3A (30' pp-lysis)	mg sRNA	Expt. 3B (60' pp-lysis)
Control sRNA	0·618	182·3	0·267	68·4	0·279	71·2	0·279	71·2
No sRNA	--	15·6	--	15·7	--	12·3	--	12·3
No sRNA, + CTP, UTP, GTP	--	17·9	--	15·2	--	--	--	--
*sRNA	0·528	22·0	0·228	50·8	0·76	56·7	0·74	27·6
*sRNA, GTP	0·528	22·2	0·228	45·0	0·76	49·4	0·74	26·2
*sRNA, CTP	0·528	24·5	0·228	75·8	0·76	93·6	0·74	76
*sRNA, UTP	0·528	28·7	0·228	52·8	0·76	53·7	0·74	29
*sRNA, CTP, GTP	0·528	22·6	0·228	76	0·76	87·9	0·74	73·7
*sRNA, UTP, GTP	--	--	0·228	46·3	0·76	48·8	0·74	29·7
*sRNA, CTP, UTP	0·528	29·8	0·228	72·3	0·76	97·6	0·74	78·8
*sRNA, CTP, UTP, GTP	0·528	22·1	0·228	79·4	0·76	87·9	0·74	70

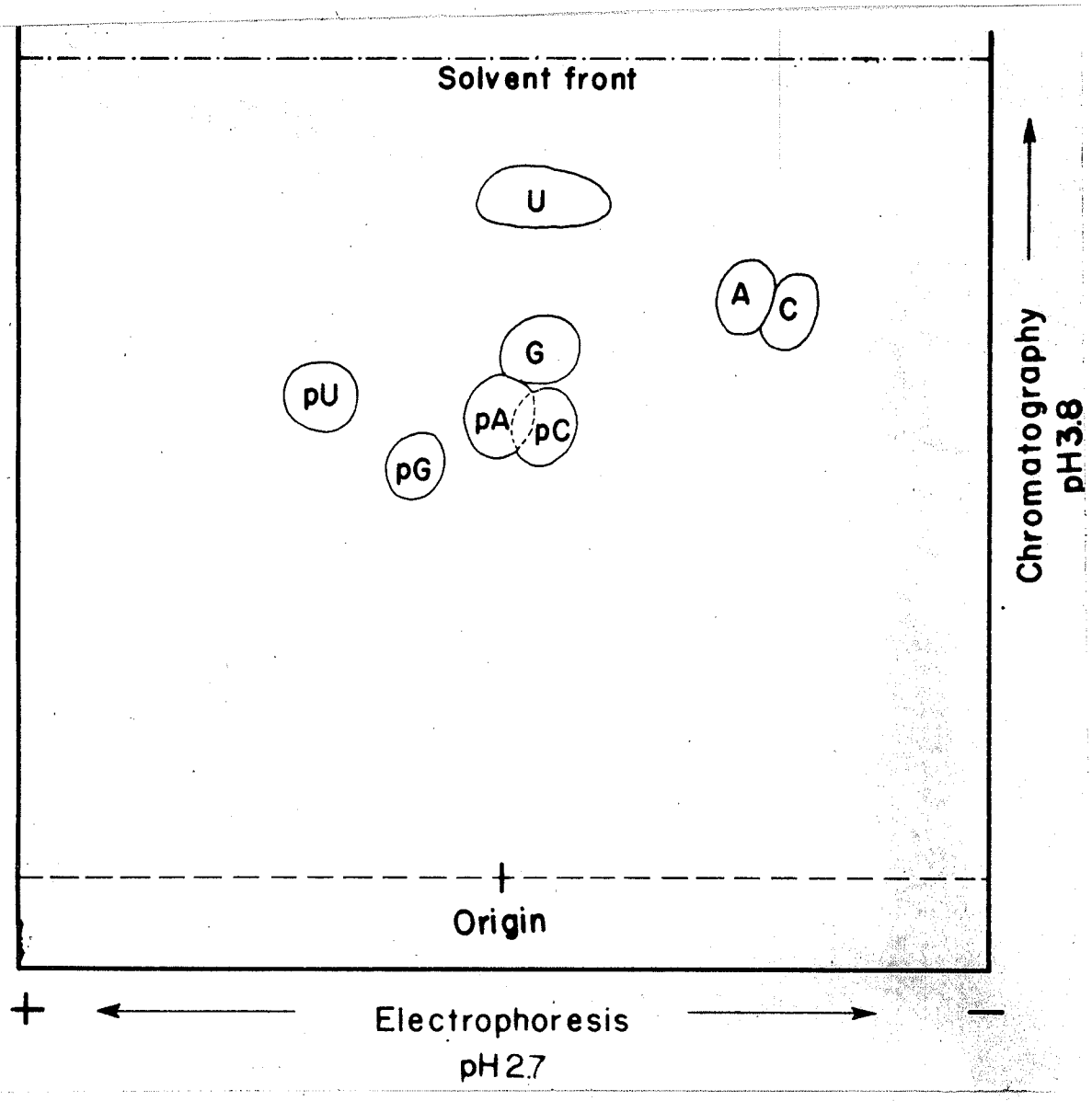
*All these sRNAs were partially pyrophosphorolyzed according to the procedure described in Materials and Methods. The control sRNA is the phenol-extracted sRNA from fresh "pH 5 enzymes" fraction without any pre-incubation. All c.p.m. expressed were corrected for background. The conditions for reconstitution of glycine acceptor activity of partially pyrophosphorolyzed sRNA were essentially the same as above. Nucleotide triphosphates were present in a final concentration of 1 μmole/ml incubation. In the case with no pre-incubation (Expt. 1) the mixtures were incubated with 0·05 mls C^{14}-glycine (12·9 mM) in the presence of 0·1 ml pH 5 enzymes at 37°C for 10 minutes. The samples were washed by the TCA washing procedure as described previously.

fraction. With a fresh RNase preparation (0·0001 μg/ml) only 3·5% glycine acceptor activity remained after RNase treatment (101). Upon the addition of CTP and in the presence of ATP a significant restoration (25%) of glycine acceptor activity was achieved (see Table 7). However, only 76% of the glycine acceptor activity was obtained in the presence of all four nucleoside triphosphates. When an aged RNase preparation (stored frozen for about a week as a 200 μg/ml solution) was used, about 62% glycine acceptor activity remained. In this case complete restoration of the glycine acceptor activity was obtained in the presence of all four nucleoside triphosphates. With a higher concentration of the same aged RNase (0·001 μg/ml), only 14·7% of the glycine acceptor activity remained. Complete reconstitution was obtained, however, in the presence of all four nucleoside triphosphates as shown in Table 7.

Reconstitution of partially pyrophosphorolyzed sRNA

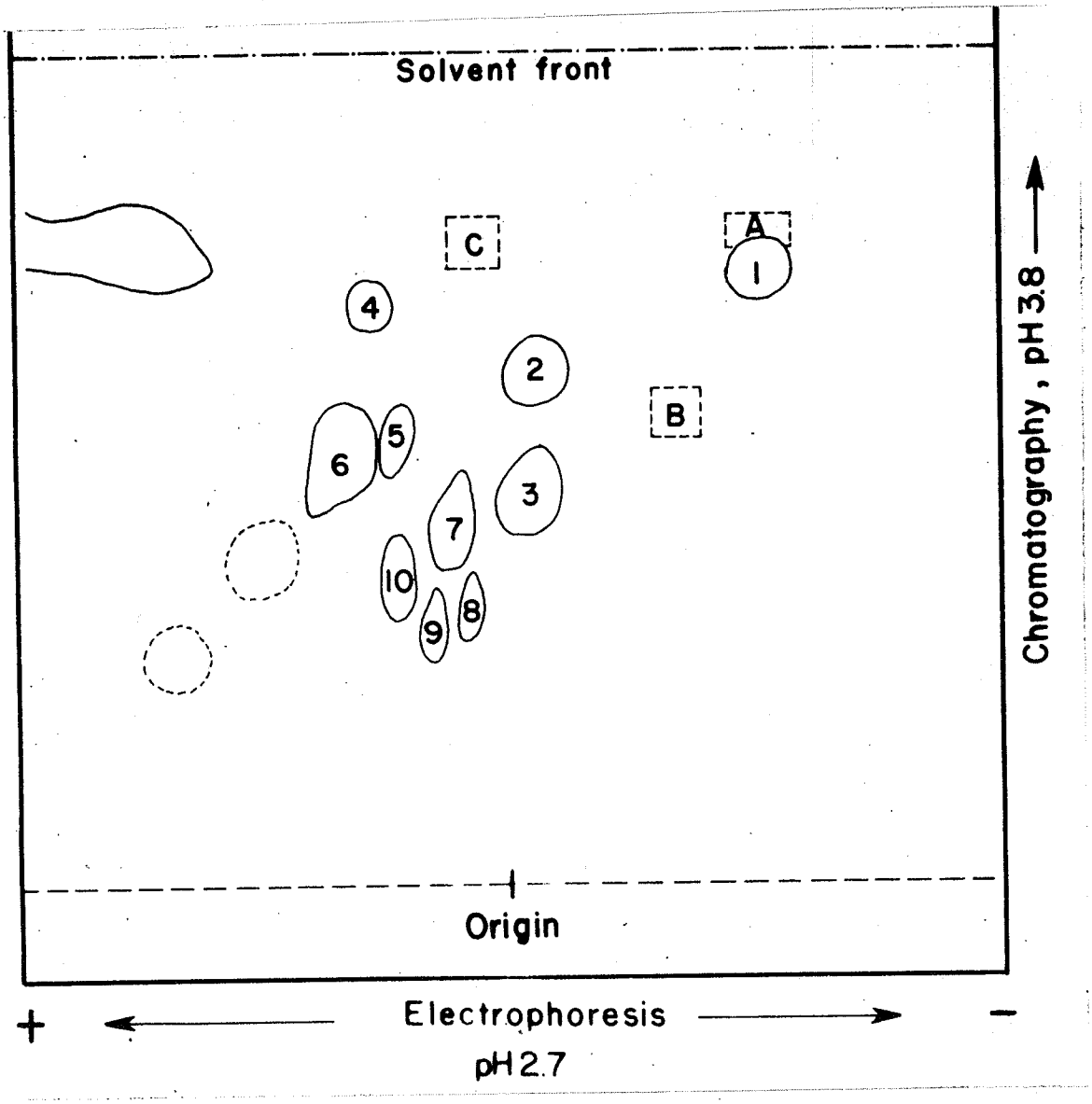
As shown in Table 8, the glycine acceptor activity of the partially pyrophosphorolyzed sRNA was restored in the presence of nucleoside triphosphates when incubated with the "pH 5 fraction". Without pre-incubation of the pyrophosphorolyzed sRNA with the nucleoside triphosphates, only a small incorporation of l-C^{14}-glycine into sRNA above the control level was obtained. When the pyrophosphorolyzed sRNA was pre-incubated with the "pH 5 enzymes" fraction in the presence of nucleoside triphosphates, significant incorporations above the control were obtained. It may be seen in Table 8 that only CTP and ATP were required to restore the glycine acceptor activity of the partially pyrophosphorolyzed

Fig. 18



Finger-print of standard nucleosides and nucleotides. Electrophoresis was carried out in ammonium formate buffer, pH 2.7, at a constant voltage of 1,500 volts at room temperature with water cooling for one hour. Chromatography was carried out in a buffer system containing tert-butanol:H O:formic acid(100:99.7:0.3), pH 3.5, at room temperature for 16 hours. pA, pC, pG and pU represent 5'monophosphates of adenosine, cytidine, guanosine and uridine respectively. A, C, G & U represent adenosine, cytidine, guanosine and uridine respectively.

Fig. 19



Finger-print of pancreatic RNase digest of acetylated 1-C^{14}-glycine-labeled sRNA
Electrophoresis was carried out in ammonium formate buffer pH 2.7, at a constant voltage of 1,500 volts at room temperature with water cooling for one hour.
Chromatography was carried out in a buffer system containing ter-butanol:H_2O:formic acid (100:99.7:0.3) pH 3.5 at room temperature for 16 hours.
The two unlabeled spots (broken line) were found in one experiment only.

Table 9

Analytical Data of Standard + Unknown Substances

Sample	R_f*	Distance moved** from origin on electrophoresis cm
Adenosine	0·69, 0·707	10·1
Guanosine	0·63	2·05
Cytidine	0·68	12·7
Uridine	0·805	1·12
5'AMP	0·55	0·635
5'GMP	0·48	3·82
5'CMP	0·54	1·9
5'UMP	0·58	8.64
Glycine	0·59	7·3
N-acetylglycine	0·755	1·58
Spot 1	0·68, 0·72	10·9
Spot 2	0·755	1·75, 1·79
Spot 3	0·45, 0·49	0·76, 1·0

*chromatography in solvent system of 100 ml tertiery butanol, 99·7 water 0·3 ml 98% formic acid at room temperature.

**electrophoresis in buffer pH 2·7 at 1,500 volts for one hour at room temperature.

Table 10

Distribution of radioactivity of spots results from finger-print of pancreatic RNase digest of acetylated C^{14}-glycine labelled sRNA (see also Fig. 19)

Spots	Radioactivities c.p.m. (corrected for background)			
	Expt. 1 (350 c.p.m.)*	Expt. 2 (538 c.p.m.)*	Expt. 3** (538 c.p.m.)*	Expt. 4 (380 c.p.m.)*
A	--	--	53 (10%)	--
B	--	--	77·8 (14·5%)	--
C	--	--	8 (1·5%)	--
1	53 (10%)	5·3 (1%)	3·5	31·4 (8·3%)
2	2·8	--	--	--
3	395 (74·5%)	515 (96%)	16·3 (3%)	208 (55%)
4	1·4	--	--	--
5	41 (7·7·5%)	4·3	--	--
6	10·8 (2·05%)	--	--	--
7	9·6 (1·8%)	--	--	--
8	10·5 (2·0%)	--	--	--
9	7·0 (1·3%)	--	--	--

*These figures represent the amount of radioactivity put on the papers. In experiment 1 the total c.p.m. found in the spots were taken as a total in calculation of the % of radioactivity in each spot.

**The RNase digest of the acetylated C^{14}-glycine labelled sRNA used in Experiment 3 was the same preparation as that used in Experiment 2 but the electrophoresis was performed five days later and seven thereafter for chromatography.

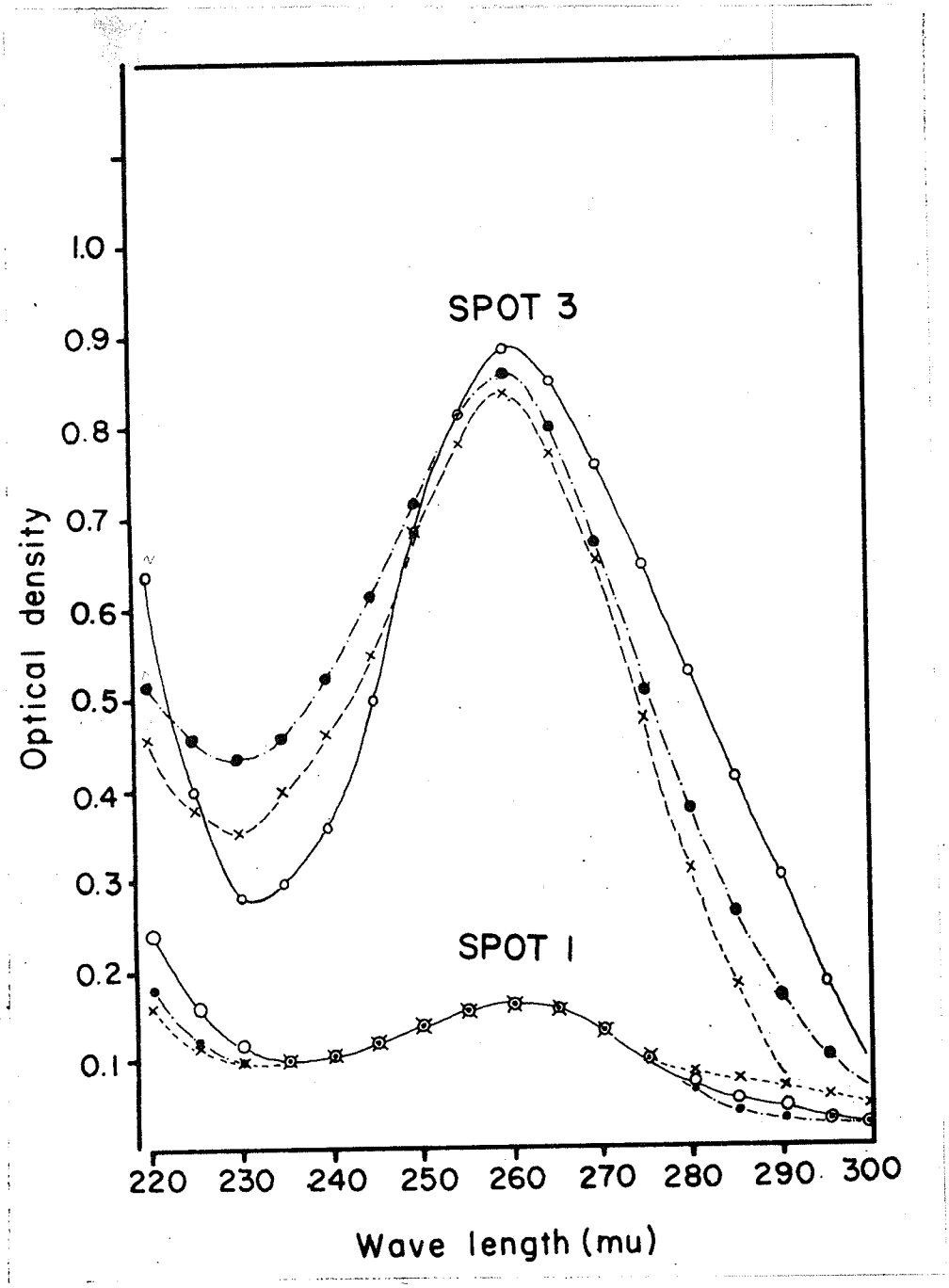
sRNA (77, 112). Neither the presence of UTP nor of GTP significantly stimulated the glycine acceptor activity.

Identification of the terminal nucleotides at the amino acid acceptor end of glycyl RNA

Fig. 18 shows a diagrammatic representation of a finger-print of standard nucleosides and nucleotides. Cytidine and adenosine were found to migrate towards the cathode whereas UMP and GMP were found to migrate towards the anode on electrophoresis at pH 2·7. Uridine, guanosine, AMP and CMP were not found to migrate from the line of origin to any great extent. On chromatography, all four nucleosides were found to be more mobile than the nucleotides under the conditions used. It may be seen from the finger-print pattern that AMP and CMP were not well separated and slightly overlapped under these conditions. Adenosine and cytidine were found on the finger-print close together, but not overlapping. The Rf values and the distances moved from the origin on electrophoresis of these nucleosides and nucleotides are given in Table 9.

Fig. 19 shows the results (presented diagrammatically) of a finger-print of a pancreatic RNase digest of acetylated 1-C^{14}-glycine-labelled sRNA (see Materials and Methods). It may be seen that Spot 1 was found in the position corresponding approximately to those of adenosine and cytidine. The three rectangular spots (broken line) on the diagram labelled A, B and C were not U.V. absorbing materials. Their relative positions in the finger-print pattern correspond to N-acetyl glycine which was released from the N-acetyl glycine ester of adenosine (A)

Fig. 20



Absorption spectra of Spot 1 and Spot 3 (see Fig. 19)

o—o at pH 2

●—● at pH 7

×—× at pH 12

during chromatography, standard free glycine (B) and standard free N-acetyl glycine (C). All other U.V. absorbing spots are believed to be di-, tri- or oligo-nucleotides which result from the attack on the acetylated 1-C^{14}-glycine-labelled sRNA by the RNase. The radioactivities of these spots were determined after elution from the paper. The results are given in Table 10. It may be seen that most of the radioactivity (63-97%) was found in spots 1 and 3 (Table 10, experiments 1, 2, 4), while other spots contained only a small amount radioactivity. In one experiment (Table 10, experiment 3) the radioactivity was found mostly in spots A and B. It should be noted that the RNase digest of the acetylated 1-C^{14}-glycine-labelled sRNA used in experiment 3 was the same preparation as that used in experiment 2, but the electrophoresis was carried out about five days later and seven days thereafter for chromatography.

Since most radioactivity was found in spots 1 and 3, these two spots were further studied. The absorption spectra of spots 1 and 3 at pH 2, 7 and 12 were determined. The results are presented in Fig. 20. The absorption spectra of spot 1 measured at the three pHs were found to be similar to that of standard adenosine, whereas the absorption spectra of spot 3 were found to be close to the absorption spectra of a standard mixture of CMP and AMP containing CMP and AMP in a molar ratio of 1:1. The absorption spectra for standard mixtures of CMP and AMP in different molar ratios are not shown, but their absorption ratios (at 260/280 and 260/230 mμ) are given in Table 11 and compared with same ratios for Spot 3. It may be seen that the absorption

Table 11

Comparison of absorption maxima, minima and 260/280, 260/230 ratios of Spot 3, CMP and AMP mixtures

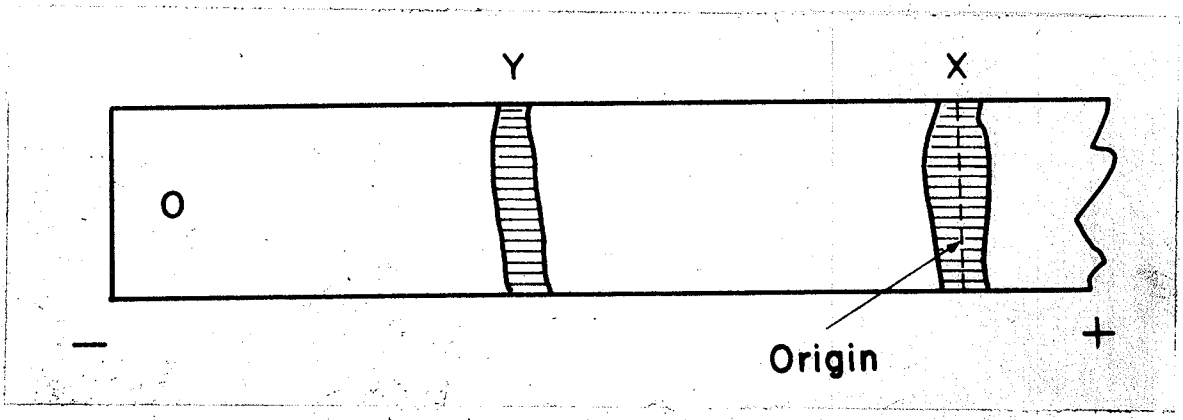
Sample		pH 2	pH 7	pH 12
Spot 3 Exp. 1	Absorption maxima	260	260	260
Exp. 2*		261	260	260
CMP:AMP 1:1		263	261	260
CMP:AMP 1·4:1		265	261	260
CMP:AMP 2:1		269	262	262
CMP:AMP 2·28:1		270	263	263
Spot 3 Exp. 1	Absorption minima	233	228	230
Exp. 2		232	230	230
CMP:AMP 1:1		235	227	230
CMP:AMP 1·4:1		235	228	230
CMP:AMP 2:1		235	228	231
CMP:AMP 2·28:1		235	230	232
Spot 3 Exp. 1	260/280 ratio	1·65	3·2	2·94
Exp. 2		1·60	2·29	2·86
CMP:AMP 1:1		1·48	2·64	2·74
CMP:AMP 1·4:1		1·24	2·2	2·34
CMP:AMP 2:1		1·06	2·05	2·06
CMP:AMP 2·28:1		0·87	1·79	1·95
Spot 3 Exp. 1	260/230 ratio	3·12	1·87	5·05
Exp. 2		3·18	1·98	2·38
CMP:AMP 1:1		3·06	2·22	2·16
CMP:AMP 1·4:1		2·8	1·83	1·82
CMP:AMP 2:1		2·46	1·68	1·62
CMP:AMP 2·28:1		2·04	1·48	1·52

Samples were dissolved in pH 2 HCl solution. After measuring the O.D. at pH 2, the samples were adjusted to pH 7 and then to pH 12 by the addition of small amount of 0·1 N KOH.

*Absorption spectra for this sample are given in Fig. 20.

Fig.21

Pattern of paper-strip electrophoresis of alkaline hydrolysate of Spot 3 (see Fig.19)



Electrophoresis was carried out in 0.02M Na-citrate buffer, pH 3.5, by the application of a constant voltage of 500 volts at room temperature for 3 hours.

Table 12

Molar ratio of X and Y

Bands	Volume of eluates	O.D. at pH 2	Calculations	
			moles	ratio
X (as CMP)	2.5 mls	0.153 (280 mu)	11.8	X : Y = 1.26 : 1.0
Y (as Adenosine)	2.5 mls	0.142 (257 mu)	9.4	

Molar extinction coefficients used in calculations were those as below (113):

Adenosine , $a_M = 15.1 \times 10^3$ at 257 mu (pH 2)

CMP , $a_M = 13.0 \times 10^3$ at 280 mu (pH 2)

maxima and minima of spot 3 measured at the three pHs are not very much different from the corresponding standard CMP:AMP mixtures. However, the 260/280 mμ absorption ratios of standard CMP:AMP mixtures show that the absorption ratios measured at the three pHs decrease significantly as the ratio of CMP:AMP increases. The same is true of the 260/230 mμ absorption ratios of standard CMP:AMP mixtures measured at the three pHs. It may be seen that the 260/280 mμ absorption ratios of spot 3 measured at the three pHs were found to be closer to the standard CMP:AMP mixture having a molar ratio of 1:1. The 260/230 mμ absorption ratios of spot 3 measured at pHs 2 and 7 were also found to be close to the standard 1:1 CMP:AMP mixture. However, the 260/230 mμ absorption ratio of spot 3 at pH 12 was found to differ significantly from those of standard CMP:AMP mixtures.

The nucleotide composition of the material contained in spot 3 was determined by eluting this spot from 6 finger-prints, pooling the samples, subjecting part of the combined material to alkaline hydrolysis. An amount of U.V. absorbing material corresponding to four spots was hydrolyzed with alkali while the remainder was used for radioactivity measurements (see Table 10, experiment 4). The alkaline hydrolysate was then analyzed by paper-strip electrophoresis as described in Materials and Methods. The results are presented in Fig. 21. Only two U.V. absorbing bands were found. One, designated as X, did not migrate appreciably from the origin, whereas the other U.V. absorbing band (designated as Y) migrated towards the cathode

(opposite to the direction of migration for nucleotides, see Fig. 4). The positions of bands X and Y correspond closely to the positions of standard CMP and adenosine. No other observable U.V. absorbing bands in the paper strip in the positions corresponding to AMP, UMP or GMP were detected. The absorbing spectra of the eluates from X and Y of the paper strip were measured at pH 2·0. It was found that the absorption spectrum of the more mobile band Y corresponded to that of adenosine, while the absorption spectrum of the eluate from band X corresponded to CMP. By using the molar extinction coefficients (113) for adenosine, $a_M = 15{\cdot}1 \times 10^3$ at 257 mμ (pH 2·0) for band Y and for CMP, $a_M = 13{\cdot}0 \times 10^3$ at 280 mμ (pH 2·0) for band X, the amount of materials in bands X and Y could be calculated. The results of this calculation show that Spot 3 contained CMP and adenosine in a molar ratio of 1·26:1 (see Table 12). Thus, Spot 3 could contain CpCpA-acetylglycine as well as CpA-acetylglycine. This implies that the fourth nucleotide at the acceptor end of glycyl RNA is Py, but not Pu.

DISCUSSION OF RESULTS

The results of the determinations of base ratios of rat liver sRNA obtained by both paper-strip electrophoresis and chromatography on Dowex 1-Cl^- column are in agreement with the values in the literature (see Table 1). The near equivalence of complementary bases is in accord with the suggestion that considerable base-pairing exists in sRNA molecules. No attempts were made in the present work to separate and identify the minor bases which are reported to occur in sRNA.

The incorporation of 1-C^{14}-glycine into rat liver sRNA (Fig. 5) was proportional to the concentration of sRNA (mg/ml) present in the incubation mixture. The results also show that the recovery of the labelled sRNA was different for different washing procedures. The specific activity (counts/min/mg) of labelled sRNA obtained by the TCA-washing procedure was found to be lower (22%) than that obtained by the washing procedure of Berg et al (74) (see Fig. 5). The poor recovery of radioactivity by the TCA-washing procedure is probably due to incomplete precipitation of the labelled sRNA. The poor recovery of labelled sRNA in some cases gives rise to a serious problem in calculation of the purification of sRNA, particularly when small amounts of sRNA are involved. The washing procedure of Berg et al had advantages over that of TCA-washing procedure, especially when partially purified glycyl RNA synthetase was used in the incubation mixture. When sRNA and the glycyl RNA synthetase were used in the form of "pH 5 enzymes", the labelled sRNA, isolated by the phenol method of Kirby (102), showed an absorption ratio 280/260 mμ of

0·45-0·5, identical to that of phenol-extracted sRNA (unlabelled). However, it was subsequently found that when phenol-extracted sRNA was labelled with l-C^{14}-glycine in the presence of partially purified glycyl RNA synthetase (50-60% $(NH_4)_2SO_4$ fraction), the labelled material, re-isolated by the phenol method of Kirby, was found to have a lower 280/260 mμ absorption ratio (0·28-0·33). The reason for this difference is not clear at present.

Phenol-extracted sRNA prepared from rat liver "pH 5 enzymes" fraction seems to contain two distinct components as revealed by chromatography on amino-ethyl cellulose column. One component eluted with buffer (K-phosphate buffer or Na-formate buffer of Zubay), while the other eluted with alkali. The fractionations of mouse Ehrlich ascites nuclear and cytoplasmic sRNAs on AE-50 and methylated albumin columns performed by Cook (106) in this laboratory also indicated that at least two components were present in each preparation, only one of which was amino acid (glycine) acceptor RNA. The nature of the second component, eluted with alkali, has not yet been investigated. It is probably an RNA fraction derived from the ribosomal fraction. It is also possible that the second component contains other amino acid acceptor RNAs. That fraction eluted with buffer can be further fractionated into at least two peaks (see Fig. 7-8). Glycyl RNA of rat liver was found to be present in the fractions eluted with K-phosphate buffer at pHs 7·0 up to 9·0 but not in the fractions eluted at pHs below 7·0 (see Table 4 and Fig. 7-8). It may be seen from the results of chromatography of intact sRNA that glycyl RNA did not elute from the column at pH 6·0 whether 0·005 M or 0·5 M K-phosphate buffer was used (Fig. 7-8).

Under these conditions the sRNA of the fractions eluted from the column did not accept radioactive glycine (Table 4). The results of pH gradient (0·5 M K-phosphate buffer pH 6·0-8·5) chromatography of intact sRNA (Fig. 8) showed that the favourable pH for elution of glycyl RNA from the AE-50 column was the range from 7·0-9·0. The glycyl RNA-rich fraction obtained under these conditions showed a purification of about 2·6-fold (Table 4).

It is of interest to note that there may be at least two glycine acceptor activity peaks (Fig. 8, 11), one at pH about 7·2 and the other at pH 7·4-9·0. These results suggest that there may be more than one type of glycyl RNA existing in the rat liver "pH 5 enzymes" fraction. These have not been reported by other investigators. However, the conclusion that there is more then one glycyl RNA must await a more thorough investigation.

Tomlinson and Tener (97) have reported the use of 7·0 M urea to eliminate the secondary binding forces in ion exchange chromatography of polynucleotides. They observed that in the absence of urea some separation of a di-nucleotide from an RNase digest of yeast RNA on DEAE-cellulose-acetate-column occurred but the remainder of the digest was eluted in a broad band, whereas in the presence of 7·0 M urea individual peaks corresponding to mono-, di- and tri-nucleotides emerged. However, the chromatography of rat liver sRNA on AE-50 column in the presence of 7·0 M urea and 0·33-0·4 M NaCl linear gradient at pH 6·0 resulted in no observable U.V. absorbing material coming through the column (Fig. 11, 12). When the concentration of NaCl rose to 1·0 M, about 82% of the U.V. absorbing material was obtained. A second

peak (containing 4·2% U.V. absorbing material) appeared when the pH of the elution was changed to 7·5. The glycine acceptor activity in these two peaks was only enriched 1·4-fold in the first peak and 1·2-fold in the second peak. When oxidized sRNA was chromatographed on AE-50 column with 0·4-1·5 M NaCl linear gradient in 7·0 M urea at pH 6·0 the oxidized sRNA was not retained on the column (Fig. 13). In fact, 64% of the U.V. absorbing material came through the column compared to 23% in the experiment shown in Fig. 9. With the same eluting system but at pH 8·2, the U.V. absorbing material (periodate treated) emerged at the starting concentration of 0·4 M NaCl (Fig. 14). The oxidized sRNA recovered from the column did not accept radioactive glycine (Table 5, Fig. 11-13) indicating that the sRNA had been completely oxidized by periodate treatment. The reason that the oxidized sRNA was not retained on the column is probably interference of Schiff base formation (see next paragraph) by the influence of urea on the secondary structure (hydrogen bonding) of sRNA molecules.

The results of chromatography of oxidized sRNA (Fig. 9-10) using the eluting system of Zubay (45) indicate that not all oxidized sRNA was retained on the column. In fact, 23% was eluted from the column under these conditions (3·0 M Na-formate and 0·5 volume% triethenolamine, at pH 8·2, on aminoethyl cellulose column, with a flow rate of 0·33 ml/min at 0°-4°C). These results are in contrast to those obtained by Zubay, who reported that most of the oxidized sRNA was retained on the column under the conditions used (3·0 M Na-formate and 0·5 volume% triethenolamine at pH 8·2 on aminoethyl cellulose column, 70 cm high, with a flow rate of 0·1 ml/min. at 5°C). This difference is probably, in addition to the β-elimination

reaction, due to the interference with Schiff base formation. The studies of reaction rate by Zubay (45) showed that the reaction of the dialdehyde with the amino-ethyl cellulose column has a half-time at room temperature of about 0·5 hours. When the oxidized and C^{14}-glycine-labelled sRNA had been kept in the column for one hour prior to starting elution more than 6% of the U.V. absorbing material was retained on the column indicating that the formation of the Schiff base complex between oxidized sRNA and amino-ethyl cellulose was rather slow. β-elimination takes place when the Schiff base is formed. Thus, Schiff base formation and β-elimination are two of the difficulties in separation of sRNA using an AE-50 column where the principle for the separation is based on the reaction between the aldehyde group of oxidized sRNA and the primary amino group of the cellulose.

In the case of separation of glycyl RNA the extremely short half-life of glycyl RNA ester as measured by Meister et al (61) and by the present work (Fig. 15) also adds an additional difficulty in obtaining a higher purification.

A considerable amount of breakdown of the glycyl RNA ester linkage might occur from the time it was labelled until periodate oxidation even though oxidation was performed at 0·1 M Na-acetate buffer pH 5·0 (see Fig. 16). As a result of this breakdown the glycine specific sRNA which no longer carries glycine will also be oxidized by periodate. This may explain the low purification obtained by the experiment shown in Fig. 6 (see Table 2).

It is shown in Fig. 15 that the half-life of C^{14}-glycine labelled sRNA in pH 7·5 buffer at 37°C was only 8-9 minutes, in good agreement with Meister et al (61). They found that glycyl RNA in pH 7·25 buffer at 37°C had a half-life of about 8 minutes. This unusual reactivity of amino acid esters of sRNA has been attributed mainly to the influence of the protonated amino group at neutral pH. This positively charged α-amino group greatly reduces the thermodynamic stability of amino acid esters of sRNA (60). Furthermore, the presence of a cis-hydroxyl group on the ribose of the terminal residue of sRNA and the size and the chemical structure of the amino acid side chains have also been shown to play a role on the stability of the amino acyl RNA (61, 107).

Acetylation of the C^{14}-glycine-labelled sRNA has been found to stabilize the ester linkage of glycyl RNA and increase the half-life of the glycyl RNA (in pH 7·5 buffer, at 37°C) by about ten times that of unacetylated glycyl RNA (Fig. 15). The half-lives of both unacetylated and acetylated C^{14}-glycine labelled sRNAs in pH 5·0 buffer, at 37°C, were found to be much longer than those at pH 7·5 (Fig. 16).

The conditions of acetylation of sRNA do not destroy the glycine acceptor activity as indicated by the experiment shown in Fig. 17 and Table 6. sRNA, which had been put through the acetylation procedure, can accept radioactive glycine to the same extent as that of the control sRNA. The stability curve of the twice acetylated C^{14}-glycine labelled sRNA (acetylated before and after labelling) followed essentially the same course as that of the control sample under the identical conditions (Fig. 17).

The mechanism of stabilization of the amino acid esters of sRNA by acetylation (or acylation) is probably that electron withdrawal by oxygen of the carbonyl group, CH_3CO^-, makes the nitrogen of an amide a much weaker electron source than the nitrogen of an amino group. Thus introduction of an acetyl group linked to nitrogen of the amino group will normally increase the thermodynamic stability of the amino acyl RNA. It would be expected that a growing peptide chain esterified at the carboxyl end with sRNA molecule ought to be more stable, since the peptide ester is not protonated (109).

Acetylation of C^{14}-glycine labelled sRNA could not be accomplished at temperetures higher than 60°C, since it was subsequently found that at temperatures above 60°C the product of acetylation contained little radioactivity. It is probable that at such a high temperature the ester linkage of glycyl RNA was broken before it could be acetylated.

The results on the reconstitution of pancreatic RNase-treated sRNA showed that the specific activities of the labelled sRNA obtained under these conditions were very much lower than usual. The reason for this variation is not known, but may in part be due to the breakdown of ATP by ATPase (70, 114), since the mixtures (no ATP regenerating system added) were preincubated at 37°C for 20 minutes with the "pH 5 enzymes" fraction. It is also possible that sRNA was degraded during the preincubation period. However, the results do indicate that reconstitution of RNase-treated sRNA can occur. The glycine acceptor activity is seem to be highly sensitive to the action of pancreatic RNase.

It is shown in Table 8 that in the presence of ATP and CTP the percentage of reconstitution of glycine acceptor activity of the pancreatic RNase-treated sRNA was much smaller than in the presence of all four nucleoside triphosphates indicating that the sRNA had been attacked by the RNase more than removal of CpCpA end group.

Nov-elle et al (101) found that leucyl RNA of E. coli, which is one of the most resistant amino acyl-RNA to B subtilis RNase or RNase T_1 digestion, is relatively sensitive to digestion with pancreatic RNase. When sRNA was treated with 0·001 µg/ml of pancreatic RNase for 20 minutes at 37°C, they found that such RNase-treated sRNA showed only 40-47% of leucine acceptor activity. However, they have not yet investigated the sensitivity of glycyl RNA towards pancreatic RNase, nor have they shown reconstitution of the amino acid acceptor activity of pancreatic RNase-treated sRNA.

Reconstitution of pyrophosphorolysed sRNA has been studied. It has been shown that incubation of sRNA with inorganic P^{32}-pyrophosphate in the presence of a partially purified enzyme fraction (nucleotide incorporating enzymes) indicates that a pyrophosphorolysis of the sRNA occurs which results in the formation of the P^{32}-nucleoside 5'-triphosphates in the acid soluble fraction (74, 105, 112). Further evidence indicates that under limited conditions this pyrophosphorolysis results only in removal of nucleotides at the acceptor end. The results of reconstitution of pyrophosphorolysed sRNA (Table 8) showed that only CTP and ATP were required to restore the glycine acceptor activity of the partially pyrophosphorolysed sRNA.

Even when the sRNA had been pyrophosphorolyzed for one hour under the conditions used (see Table 8, experiment 3B) neither UTP nor GTP showed a significant restoring effect on the glycine acceptor activity. This is in accord with Zamecnik et al (77), who found dependence upon CTP for the binding of amino acid to sRNA of pre-incubated pH 5 enzymes fraction for the 14 natural C^{14} amino acids tested, including glycine.

These results support the observation that pyrophosphorolysis of sRNA occurs only at the amino acid acceptor end under the limited conditions. The reaction can proceed further if the mixture is incubated for a longer time or in the presence of a higher concentration of pyrophosphate. This unusual resistance of sRNA to further pyrophosphorolysis is probably due to its helical secondary structure. The susceptibility of the amino acid acceptor end towards the attack by certain enzymes has led to the suggestion (22, 57) that the acceptor end is not in the region of hydrogen bonding.

It is apparent from the result of the finger-print of pancreatic RNase digest of acetylated C^{14}-glycine labelled sRNA (Fig. 19, Table 10) that glycine is esterified to AMP of the terminal acceptor end by the action of glycyl RNA synthetase. This direct evidence for the existence of the glycine ester of adenosine in the pancreatic digest would not have been obtained if the C^{14}-glycine labelled sRNA had not been stabized by acetylation, since the half-life of the glycine ester of sRNA is only 8-9 minutes (Fig. 15). The finding that a considereble

amount of radioactivity was found in the position slightly above spot 1 (the R_f for this radioactive spot is the same as that of free N-acetyl glycine, see Fig. 19, spot A,(Table 10))is considered as an indication that degradation of the acetylated glycine ester of adenosine had been occuring during the course of chromatography.

The results also suggest that pancreatic RNase attacks the sRNA randomly (101) since both CpA-acetyl glycine and adenosine-acetyl-glycine were present (Fig. 19, 20) in the RNase digest under the conditions used. However, it is not clear whether the acetylated glycine, which was esterified to the terminal residue of AMP, will cause the RNase attack preferentially on CpA acetyl glycine rather than on adenosine-acetyl glycine, since more radioactivity was found in spot 3 than in spot 1 (36).

The results of paper-strip electrophoresis of the alkaline hydrolysate of spot 3 showed that it was composed of CMP and adenosine in a molar ratio of 1·26:1 (Fig. 21, Table 12). Comparison of absorption spectra of mixtures of standard CMP and AMP of known molar ratios with the eluate from spot 3 suggest that spot 3 was composed of CMP and adenosine in a ratio close to 1:1, in fair agreement with that obtained by analysis of the alkaline hydrolysate of spot 3. These results indicate that spot 3 must therefore be CpA.

Moreover the presence of free radioactive N-acetylglycine in the finger-print of the pancreatic RNase digest of acetylated C^{14}-glycine labelled sRNA clearly demonstrated that the C^{14}-glycine which had been esterified to sRNA enzymatically has, in fact, been acetylated by the acetylation procedure.

The finger-print pattern of the pancreatic digest (Fig. 19) and the alkaline hydrolysate (unpublished observation) of sRNA showed that adenosine was the only nucleoside found in the finger-print of rat liver sRNA hydrolysate as detected under the U.V. lamp. No detectable spots corresponding to other nucleosides could be observed. These observations are in agreement with the results of other investigators showing that adenylic acid is a terminal residue in the sRNA molecule.

It may be concluded that the terminal nucleotide sequence at the amino acid acceptor end of glycyl RNA is pPypCpA where Py represents a pyrimidine. This is in agreement with the results from reconstitution of pyrophosphorolysed sRNA and also in accord with the findings of other investigators with other amino acid acceptor RNAs.

ABBREVIATIONS

aa : amino acid;
sRNA : soluble RNA or acceptor RNA or transfer RNA;
rRNA : ribosomal RNA;
DNA : deoxyribonucleic acid;
ATP : adenosine triphosphate;
PP^{32} : radioactive inorganic pyrophosphate;
TCA : trichloroacetic acid;
PCA : perchloric acid;
cpm : counts per minute;
O.D. : optical density;
Mg^{++} : magnessium ion;
U.V. : ultra-violet;
ϵ_M : molar extinction coefficient
E_{260} : extinction coefficient at 260 mu;
RNase : ribonuclease;
pA, pC, pU and pG : 5'phosphates of adenosine, cytidine, uridine and guanosine;
AMP, CMP, UMP and GMP : 2' or 3' monophosphates of adenosine, cytidine, uridine and guanosine;
A, C, U and G : adenosine, cytidine, uridine and guanosine.

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