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Studies on Chemical Modification of Thionucleosides in the Transfer Ribonucleic Acid of *Escherichia coli*

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^{35}S-labelled tRNA from *Escherichia coli* was treated with chemical reagents such as CNBr, H_2O_2, NH_2OH, I_2, HNO_2, $KMnO_4$ and $NaIO_4$, under mild conditions where the four major bases were not affected. Gel filtration of the treated tRNA showed desulphurization to various extents, depending on the nature of the reagent. The treated samples after conversion into nucleosides were chromatographed on a phosphocellulose column. NH_2OH, I_2 and $NaIO_4$ reacted with all the four thionucleosides of *E. coli* tRNA, 4-thiouridine (s^4U), 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), 2-thiocytidine (s^2C) and 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A), to various extents. CNBr, HNO_2 and $NaHSO_3$ reacted with s^4U, mnm^5s^2U and s^2C, but not with ms^2i^6A. $KMnO_4$ and H_2O_2 were also found to react extensively with thionucleosides in tRNA. Iodine oxidation of ^{35}S-labelled tRNA showed that only 6% of the sulphur was involved in disulphide formation. Desulphurization of *E. coli* tRNA with CNBr resulted in marked loss of acceptor activities for glutamic acid, glutamine and lysine. Acceptor activities for alanine, arginine, glycine, isoleucine, methionine, phenylalanine, serine, tyrosine and valine were also affected, but to a lesser extent. Five other amino acids tested were almost unaffected. These results indicate the fate of thionucleosides in tRNA when subjected to various chemical reactions and the involvement of sulphur in aminoacyl-tRNA synthetase recognition of some tRNA species of *E. coli*.

Modification of the nucleosides with chemical reagents is one of the methods used for studying the interrelationship of structure and function of tRNA, and also the relationship between tRNA conformation and chemical reactivity of its various functional groups. Chemical reagents have been used to distinguish single-stranded exposed nucleotides from those that are in helical complexes or otherwise protected from reaction. As discussed by Kochetkov & Budowsky (1969), the action of a chemical reagent on nucleic acids usually results in simultaneous modifications of several types of nucleoside units. Thus it is difficult to obtain a polymer with chemical alterations of known extent and character. But this need not be true with the chemical modification of minor bases such as thio-bases in tRNA, because the particular class of minor bases present in a tRNA species are limited in number and also they are highly reactive. Thus Walker & RajBhandary (1972), after modifying *Escherichia coli* $tRNA^{Met}$ with CNBr and analysing the ribonuclease T_1 digests of the modified tRNA, found that only the s^4U* moiety of the tRNA was affected and the acceptor activity unchanged. Since the discovery of the rare nucleoside, s^4U, in the tRNA of *E. coli* (Lipsett, 1965), several investigations have been carried out into its chemistry and biological function. s^4U in *E. coli* tRNA is characterized by its presence in the eighth position from the 5′ end of the tRNA molecule (Dirheimer *et al.*, 1972), greater acidity, ease of oxidation and dimer formation (Lipsett, 1967; Yaniv *et al.*, 1969). The desulphurization, apart from dimerization, is the most widely used chemical modification of s^4U. Several chemical reagents, such as CNBr (Saneyoshi & Nishimura, 1970; Walker & RajBhandary, 1970), H_2O_2 (Scheit, 1968), NH_2OH (Iida *et al.*, 1973), HNO_2 (Iida & Hayatsu, 1971), $KMnO_4$ (Yano & Hayatsu, 1970), $NaHSO_3$ (Hayatsu & Inoue, 1971) and $NaIO_4$ (Ziff & Fresco, 1968) have been used to convert s^4U into U. The reactions of s^4U with some of these reagents have been shown only at the monomer level and thus its reactivity as a constituent of tRNA is not known.

In addition to s^4U, *E. coli* tRNA is known to contain three more thionucleosides, i.e. mnm^5s^2U, s^2C (Carbon *et al.*, 1968), and ms^2i^6A (Burrows *et al.*, 1968). The fate of these three thionucleosides when s^4U is involved in a reaction is not well documented. Hecht *et al.* (1971) have reported the use of Raney nickel to desulphurize ms^2i^6A specifically in *E. coli* tRNA without affecting much s^4U. We report here the effect of different chemical reagents on the four

* Abbreviations: s^4U, 4-thiouridine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; s^2C, 2-thiocytidine; ms^2i^6A, 2-methylthio-N^6-isopentenyladenosine; Q and N, unidentified minor nucleosides; RNAase, ribonuclease.

thionucleosides of *E. coli* tRNA and the effect of removal of sulphur on the amino acid-acceptor activity of tRNA.

Materials and Methods

Materials

Carrier-free $H_2{}^{35}SO_4$ was obtained from Bhabha Atomic Research Centre, Bombay, India. Phosphocellulose, peptone, casamino acids, beef extract and yeast extract were from Vallabhai Patel Chest Institute, Delhi, India. Alkaline phosphatase was from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and snake-venom phosphodiesterase, pancreatic RNAase and PPO (2,5-diphenyloxazole) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Labelled amino acids were from Bhabha Atomic Research Centre and The Radiochemical Centre, Amersham, Bucks., U.K. All the other chemicals used were purchased commercially and were of reagent grade.

Growth of bacteria and isolation of tRNA

The growth of *E. coli* in a minimal medium containing [^{35}S]sulphate and the isolation of the labelled tRNA were as described (Rao & Cherayil, 1973; von Ehrenstein, 1967). Non-radioactive tRNA and the aminoacyl-tRNA synthetase were prepared from bacteria grown in a nutrient medium. The nutrient medium (pH7.2) contained (per litre) peptone (5g), NaCl (3.5g), beef extract (1.5g) and yeast extract (1.5g). For enzyme preparation the cells were harvested in mid-exponential phase.

Preparation of nucleotides and nucleosides

Enzymic digestion of tRNA to mononucleotides was performed by digesting 1mg of tRNA in 1ml with 300μg of snake-venom phosphodiesterase in NH_4HCO_3 buffer (pH8.9) at 37°C for 18h. Alkaline digestion of tRNA was carried out with 0.3M-KOH at 37°C for 18h and the nucleotides were converted into nucleosides by digestion with alkaline phosphatase (50μg/mg of tRNA) in 0.1M-Tris–HCl buffer, pH8.8, for 16h at 37°C.

Column-chromatographic methods

Phosphocellulose column chromatography of nucleosides was performed as described by Rao & Cherayil (1973). Separation of mono- and di-4-thiouridylate on a DEAE-cellulose column was done by the method of Lipsett (1967). Sephadex G-100 gel filtration was carried out on a 1.3cm×40cm column with 0.1M-NaCl in 0.01M-Tris–HCl (pH7.5) as solvent.

tRNA charging

tRNA was aminoacylated (Cherayil *et al.*, 1968) by incubation at room temperature (23°C) for 30min, on a filter-paper disc containing 1 E_{260} unit of tRNA, a mixture containing, in 0.15ml, 50mM-potassium maleate (pH7.2), 5mM-$MgCl_2$, 0.5mM-EDTA, 5mM-2-mercaptoethanol, 1mM-ATP, 0.5mM-CTP, 0.1μCi of ^{14}C-labelled amino acid (1–2nmol) or 1μCi of ^{3}H-labelled amino acid (0.2–2nmol) and about 0.5mg of protein of crude *E. coli* aminoacyl-tRNA synthetase enzyme. The crude enzyme was obtained by adsorbing a 105000*g* supernatant on a DEAE-cellulose column and eluting it with 0.3M-KCl (Muench & Berg, 1966). At the end of the incubation, the filter-paper discs were partially dried in hot air and put into 10% (w/v) trichloroacetic acid containing 0.2% casamino acids. Then the paper discs were washed several times in a Büchner funnel with 5% trichloroacetic acid followed by ethanol and dried and counted for radioactivity in a Beckman LS-100 liquid-scintillation counter with 0.5% (w/v) PPO in toluene as the scintillation fluid.

Chemical modifications of ^{35}S-labelled tRNA

(*i*) *Cyanogen bromide.* The tRNA was modified with CNBr by the method of Saneyoshi *et al.* (1972). The thiocyanato derivatives of tRNA were prepared by treating 40 E_{260} units of tRNA in 1ml of 0.05M-sodium phosphate buffer, pH8.6, with 0.5mg of CNBr in 0.3ml of ethanol for 10min. The thiocyanato derivatives of tRNA were hydrolysed to the corresponding oxy derivatives by keeping in 0.1M-sodium acetate buffer, pH4.7, at 37°C for 12h.

(*ii*) *Hydrogen peroxide.* The treatment of tRNA with H_2O_2 was performed as outlined by Scheit (1968) and adapted by Shugart (1972). For this 10μl of 30% (v/v) H_2O_2 was mixed with 20 E_{260} units of tRNA in 1ml of 0.1M-Tris–HCl buffer, pH8.0, and the reaction was allowed to proceed at 23°C for 90min. The tRNA was recovered by precipitation with 2vol. of ethanol.

(*iii*) *Hydroxylamine.* Modification of tRNA with NH_2OH was done as outlined by Iida *et al.* (1973) for the modification of the nucleoside 4-thiouridine. Among the various conditions described by them, conditions under which the common nucleosides were unaffected or least affected were chosen. The tRNA (40 E_{260} units/ml) in 0.2M-Tris–HCl, pH7, was treated with 0.5M-NH_2OH at pH7.0 and 37°C for 35min. NH_2OH solution was prepared fresh by dissolving NH_2OH,HCl in 0.2M-Tris–HCl, pH7.0, and adjusting the pH to 7.0 with NaOH. After the reaction the modified tRNA was recovered by repeated ethanol precipitation.

(*iv*) *Iodine.* The oxidation of tRNA with iodine was carried out by the method of Carbon *et al.* (1965).

(*v*) *Nitrous acid.* The method described by Iida & Hayatsu (1971) for the conversion of s^4U was adapted for the treatment of tRNA. The reaction mixture contained freshly prepared 0.09M-$NaNO_2$ in 0.2M-sodium acetate buffer, pH adjusted to 4.9 with acetic acid, and 4mg of tRNA/ml (88 E_{260} units). The incubation was carried out for 5h at 37°C, after which the tRNA was recovered by repeated ethanol precipitation.

(*vi*) *Potassium permanganate.* The tRNA was oxidized with $KMnO_4$ by adapting the method described by Yano & Hayatsu (1970) for the oxidation of the nucleoside 4-thiouridine. The tRNA (5mg/ml) was treated with 0.1mM-$KMnO_4$ at pH7.0 and 0°C for 6min in 0.1M-sodium phosphate buffer. After the reaction the extra permanganate was destroyed by adding 3mM-ascorbic acid. The tRNA was then recovered by ethanol precipitation. The hydrolysis of sulphonates in tRNA was performed by incubating the $KMnO_4$-treated tRNA at pH4.0 in 0.1M-sodium acetate buffer for 180min at room temperature.

(*vii*) *Sodium bisulphite.* The reaction was carried out in a solution of 80 E_{260} units of tRNA/ml in 2M-$NaHSO_3$, pH5.8, at room temperature for 24h (Singhal, 1971). After the incubation, the solution was diluted and adsorbed on a small column of DEAE-cellulose. Then the bisulphite was washed away with 0.1M-Tris–HCl (pH7.4) and the tRNA was eluted with 1M-NaCl–0.1M-Tris–HCl (pH7.4) and recovered by precipitation with ethanol.

(*viii*) *Sodium periodate.* The tRNA solution (3–5mg/ml) was treated with 0.1M-sodium metaperiodate (freshly prepared) at room temperature for 30min in the dark (Hayward & Weiss, 1966). The excess of periodate was then removed by the addition of 0.1 vol. of 2M-KCl and centrifuging out the precipitated KIO_4. The tRNA from the supernatant was recovered by precipitation with ethanol.

Results

Except for the bisulphite modification, conditions used for the modification of tRNA were such that the major nucleosides are known to be unaffected or least affected. The ^{35}S-labelled tRNA was treated with various chemical reagents and a sample was gel-filtered to see the effect of the reagent on the sulphur in tRNA. Appropriate controls were also run without the reagent. The chemically treated tRNA was then converted into nucleosides by hydrolysis with alkali followed by treatment with phosphatase and chromatographed on a phosphocellulose column to find the effect of the reagent on individual thionucleosides. Quantitative data of the results of chemical modification are in Tables 1 and 2.

The ^{35}S-labelled tRNA of *E. coli*, prepared as described in the Materials and Methods section, was eluted as a single peak when gel-filtered on Sephadex G-100, the radioactivity coinciding with the absorbance and being RNAase-sensitive (Fig. 1*a* and 1*b*). The gel-filtration patterns obtained after treatment of ^{35}S-labelled tRNA with various reagents are shown in Fig. 1 (*c–f*) and Fig. 2 (*a–f*). Reactions with CNBr and $KMnO_4$ resulted in the loss of 30 and 15% of the sulphur respectively (Figs. 1*c* and 2*c*). The thiocyanato derivatives formed by CNBr treatment, when incubated at pH4.7, released 60% more of the sulphur (Fig. 1*d*). Likewise the thiosulphonates formed after treatment with $KMnO_4$, when incubated at pH4.0, resulted in the loss of about 32% more of the sulphur (Fig. 2*d*). Thus CNBr reacted with 90% of the sulphur and $KMnO_4$ with 47% of the sulphur present in *E. coli* tRNA.

Table 1. *Desulphurization of E. coli ^{35}S-labelled tRNA by various chemical reagents*

The ^{35}S-labelled tRNA was treated with various reagents as described in the Materials and Methods section and gel-filtered on a column of Sephadex G-100 and the amount of sulphur removed from the tRNA was noted. Appropriate controls were also run for each experiment by omitting the reagent. The chemically modified tRNA, after conversion into nucleosides and chromatography on a phosphocellulose column, was found to lose sulphur more than the control tRNA. This value has been taken as sulphur labilized by the reagent to alkali treatment.

Reagent	tRNA desulphurized (%)	Sulphur labilized (%)	Total sulphur removed or reacted (%)
CNBr	30	60	90
H_2O_2	80	12	92
NH_2OH	20	56	76
I_2	24	21	45
HNO_2	68	0	68
$KMnO_4$	15	35	50
$NaHSO_3$	51	17	68
$NaIO_4$	71	27	98

Table 2. *Effect of chemical modification on the thionucleosides of E. coli tRNA by various reagents*

Percentage of thionucleosides remaining were shown by phosphocellulose chromatography. For full details see the text. ?, not known.

Chemical modification	Percentage remaining			
	s^4U	ms^2i^6A	s^2C	mnm^5s^2U
CNBr	0	100	0	0
H_2O_2	<1	50	100	0
NH_2OH	20	60	0	20
I_2	53	75	50	52
HNO_2	18	100	67	50
$KMnO_4$	40	0	?	80
$NaHSO_3$	19	100	0	60
$NaIO_4$	0	16	0	0

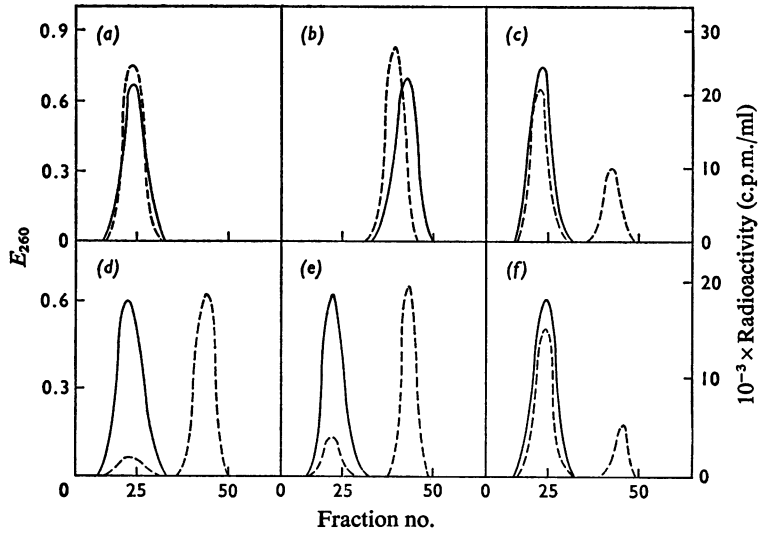

Fig. 1. *Gel filtration of ^{35}S-labelled tRNA on Sephadex G-100 after treatment with various chemical reagents*

The gel filtration was carried out on a 1.3cm × 40cm column by using a buffer containing 0.1M-NaCl and 0.01M-Tris–HCl, pH7.5. Flow rate was 20ml/h and 1.2ml fractions were collected. (*a*) Untreated ^{35}S-labelled tRNA; (*b*) ^{35}S-labelled tRNA treated with pancreatic RNAase before gel filtration; (*c*) ^{35}S-labelled tRNA treated with CNBr at pH8.6 for 10min at room temperature; (*d*) ^{35}S-labelled tRNA after reaction with CNBr as in (*c*) recovered by precipitation with ethanol and incubated at pH4.7 at 37°C for 12h; (*e*) ^{35}S-labelled tRNA treated with H_2O_2 at pH8.0 and room temperature (23°C) for 90min; (*f*) ^{35}S-labelled tRNA treated with 0.5M-NH_2OH at pH7.0 and 37°C for 35min. ——, E_{260}; ----, radioactivity.

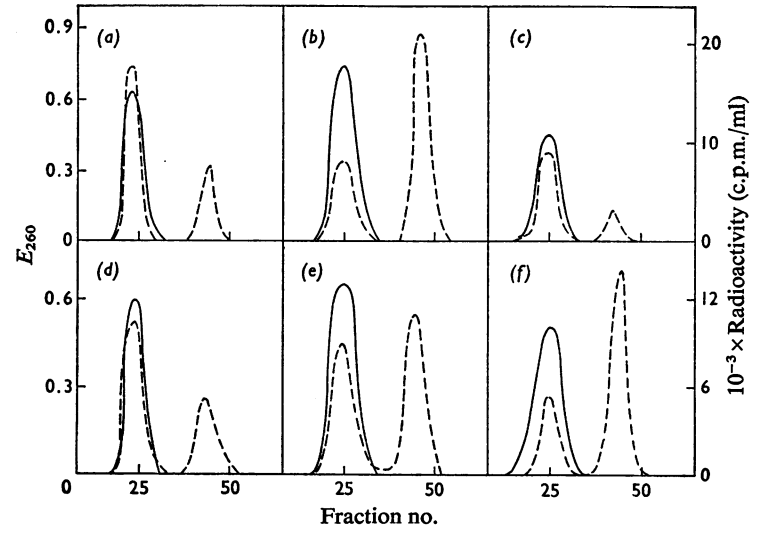

Fig. 2. *Gel-filtration patterns of ^{35}S-labelled tRNA after treatment with various chemical reagents*

The conditions used for gel filtration on Sephadex G-100 were the same as those given in Fig. 1. (*a*) ^{35}S-labelled tRNA oxidized with 0.5mM-iodine in 0.5% KI, pH7.0 for 15min at 0°C; (*b*) ^{35}S-labelled tRNA incubated for 5h at 37°C in 0.09M-$NaNO_2$ in 0.2M-sodium acetate buffer, pH4.9; (*c*) ^{35}S-labelled tRNA treated with 0.1mM-$KMnO_4$ at pH7.0 and 0°C for 6min; (*d*) $KMnO_4$-treated tRNA (from *c*) recovered by ethanol precipitation and incubated at pH4.0 for 180min; (*e*) ^{35}S-labelled tRNA incubated in 2M-$NaHSO_3$, pH5.8 at room temperature (23°C) for 24h; (*f*) ^{35}S-labelled tRNA treated with 0.1M-sodium metaperiodate at room temperature for 30min. ——, E_{260}; ----, radioactivity.

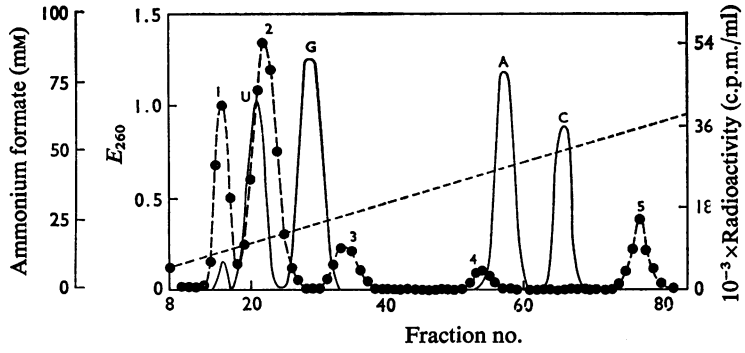

Fig. 3. *Phosphocellulose chromatography of ^{35}S-labelled thionucleosides of E. coli tRNA*

Some 28 E_{260} units of labelled nucleosides containing 900000 c.p.m. were chromatographed on a column (1 cm × 56 cm) with a linear gradient (----) formed from 125 ml each of 0.005 M- and 0.1 M-ammonium formate (pH 3.9). The flow rate was 10 ml/h and the average volume of each fraction was 2 ml. Radioactivity (●) peaks 2, 3, 4 and 5 correspond to s^4U (74%), ms^2i^6A (10%), s^2C (3%) and mnm^5s^2U (13%) respectively. Radioactivity in the first peak consisting of non-nucleoside matter amounted to 24% of the total radioactivity loaded on the column. Recovery of radioactivity from the column was more than 95%. ——, E_{260}.

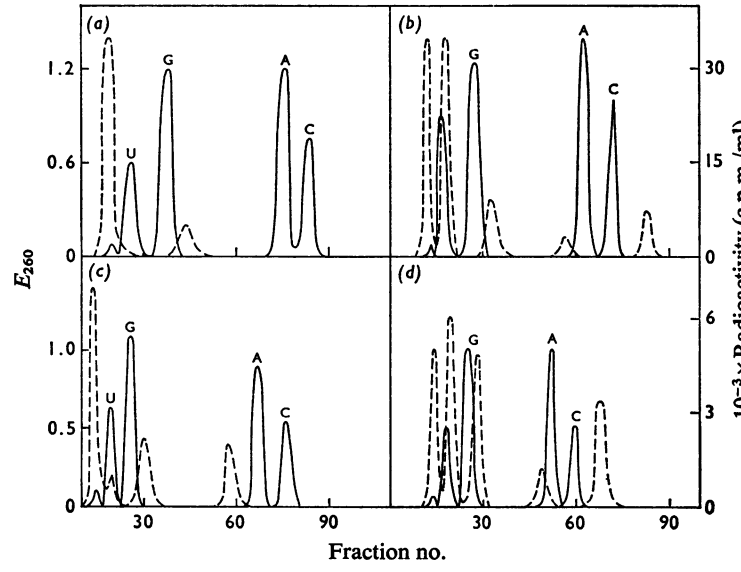

Fig. 4. *Analysis of thionucleosides from ^{34}S-labelled tRNA after treatment with CNBr (a), I_2 (b), H_2O_2 (c) and HNO_2 (d)*

^{35}S-labelled tRNA was treated with the various chemical reagents as described in the Materials and Methods section. After the reaction the tRNA was recovered by ethanol precipitation and freed from the chemical reagents by further precipitations with ethanol. The tRNA was hydrolysed to nucleosides by treatment with 0.3 M-KOH for 18 h and alkaline phosphatase for 16 h and chromatographed on a phosphocellulose column. Conditions for chromatography were the same as in Fig. 2. ——, E_{260}; ----, radioactivity.

The phosphocellulose column chromatographic separation of the ^{35}S-labelled nucleosides of *E. coli* tRNA is shown in Fig. 3. The second, third, fourth and fifth peaks of radioactivity correspond to s^4U (74%), ms^2i^6A (10%), s^2C (3%) and mnm^5s^2U (13%) respectively. The first peak of radioactivity, amounting to about 24% of the total radioactivity loaded, consists mostly of free sulphur and a small amount of nucleotide sulphur. The phosphocellulose column chromatographic patterns of the nucleosides derived

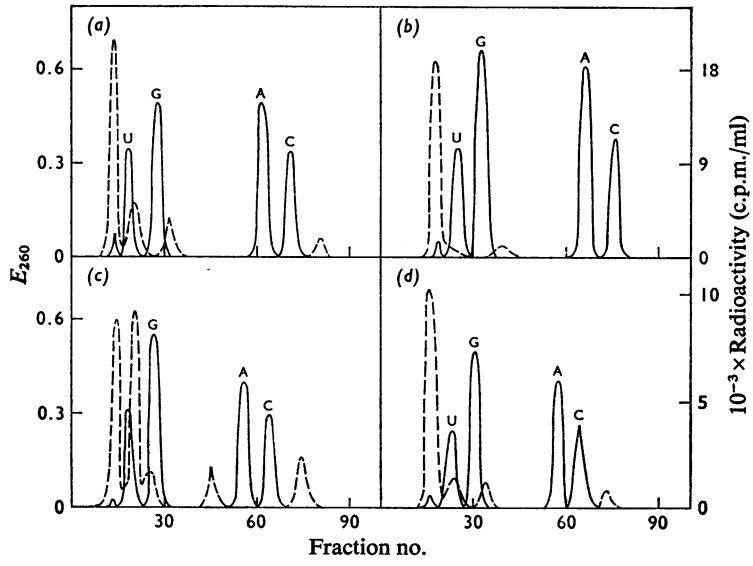

Fig. 5. *Analysis of thionucleosides from* ^{35}*S-labelled tRNA after treatment with* NH_2OH (*a*), $NaIO_4$ (*b*), $KMnO_4$ (*c*) *and* $NaHSO_3$ (*d*)

For details see the legend for Fig. 4.

from ^{35}S-labelled tRNA treated with the reagents are shown in Fig. 4 (*a–d*) and Fig. 5 (*a–d*). As can be seen the reaction with the individual thionucleosides in the tRNA varied with the reagent (Table 2).

Reaction with iodine was studied further to determine the amount of disulphide that might be formed. Iodine oxidation of the ^{35}S-labelled tRNA had resulted in 25% loss of radioactivity (Fig. 2*a*). No radioactivity was observed in the high-molecular-weight region, showing that no intermolecular disulphide-bond formation occurs in the tRNA owing to iodine oxidation. The conditions used for gel filtration were such that a dimer of tRNA would have separated from the monomer (Hampel *et al.*, 1971). Fig. 6(*a*) shows the fractionation, on a DEAE-cellulose column, of nucleotides derived from ^{35}S-labelled tRNA by alkaline hydrolysis. The iodine-oxidized tRNA was converted into nucleotides enzymically, as any disulphides formed would be hydrolysed by alkaline digestion (Uziel, 1966), and fractionated on a column of DEAE-cellulose (Fig. 6*b*). About 6% of the radioactivity appeared in the dimer position and on reduction with $Na_2S_2O_3$ the dimer peak disappeared (Fig. 6*c*).

Effect of desulphurization of tRNA with CNBr on amino acid-acceptor activity

E. coli tRNA was desulphurized by treatment with CNBr at pH8.6 and then incubation of the tRNA containing the thiocyanato derivatives at pH4.7. From Figs. 1(*c*), 1(*d*) and 4(*a*) it is clear that the above treatment removes sulphur quantitatively from the three thiopyrimidines present in the tRNA. The extent of aminoacylation of the thus desulphurized tRNA was measured with all the amino acids except cysteine, proline and tryptophan, by using excess of a crude preparation of *E. coli* aminoacyl-tRNA synthetase, and compared with that of untreated tRNA (results not shown). In addition to the thiopyrimidines in tRNA, the Q and N nucleosides are also known to be affected by CNBr (Walker & RajBhandary, 1972; Saneyoshi & Nishimura, 1970, 1971). Acceptor activities for glutamic acid, glutamine and lysine were markedly affected by the desulphurization (up to 90%), whereas tRNA species corresponding to arginine, glycine, methionine, phenylalanine, tyrosine, valine and serine lost about 35–65% of their activity. Alanine, isoleucine and threonine tRNA species retained 80% or more of their activity, and the acceptor activities for aspartic acid, asparagine, histidine and leucine were unaffected.

Discussion

The reaction of CNBr with s^4U in tRNA was first reported by Saneyoshi & Nishimura (1967). CNBr reacts with s^4U forming uridine 4-thiocyanate at near-neutral pH values, and under mild acidic or alkaline conditions (Saneyoshi *et al.*, 1972) or by

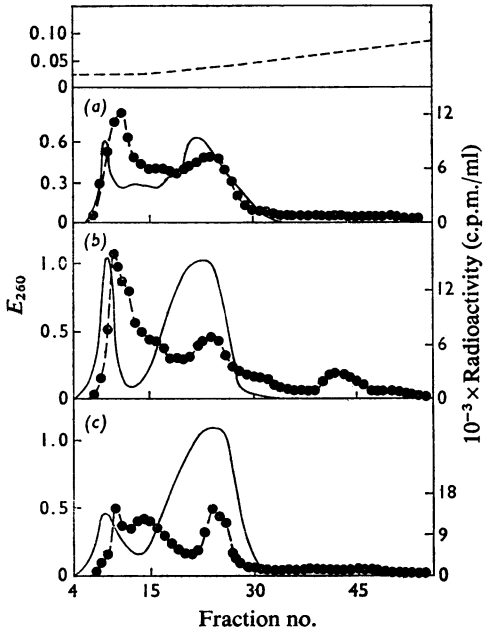

Fig. 6. *DEAE-cellulose column chromatography of* ^{35}*S-labelled nucleotides*

The chromatography was done by the method of Lipsett (1967). The samples were applied to a column (1.2cm×7cm) of DEAE-cellulose in 0.005M-sodium phosphate buffer, pH7.0, and washed with 10ml of 0.005M-sodium phosphate–7M-urea–0.025M-NaCl. Then the column was developed with a linear gradient consisting of 75ml of 0.005M-sodium phosphate–7M-urea–0.025M-NaCl, pH7.0, in the mixing chamber and 75ml of 0.005M-sodium phosphate–7M-urea–0.15M-NaCl, pH7.0, in the reservoir. Flow rate was 15ml/h and 1.8ml fractions were collected. Recovery of radioactivity was about 85%. (*a*) ^{35}S-labelled tRNA was converted into nucleotides by treatment with 0.3M-KOH for 18h at 37°C. (*b*) Iodine-treated ^{35}S-labelled tRNA was enzymically converted into nucleotides by treatment with snake-venom phosphodiesterase in NH_4HCO_3 buffer (pH8.9) at 37°C for 18h. The region between fractions 40 and 48 corresponds to dinucleotides. About 6% of the total radioactivity recovered was eluted in this region. (*c*) ^{35}S-labelled nucleotides obtained by enzymic digestion of iodine-treated ^{35}S-labelled tRNA as described in (*b*) were incubated in 0.05M-$Na_2S_2O_3$ (pH7.2) for 4h at 37°C. ----, NaCl gradient; ●, radioactivity; ——, E_{260}.

keeping at 100°C for a few minutes (Walker & Raj-Bhandary, 1970) the thiocyanato derivative is hydrolysed to uridine. Under these conditions the four major bases were found not to react with CNBr (Saneyoshi & Nishimura, 1967) Results presented here clearly show that s^4U, s^2C and mnm^5s^2U present in tRNA react quantitatively with CNBr, whereas ms^2i^6A is unaffected. Walker & RajBhandary (1972) have also observed that ms^2i^6A in *E. coli* $tRNA^{Tyr}$ was unaffected by CNBr treatment. Our results show that ^{35}S-labelled tRNA on reaction with CNBr loses about 30% of sulphur during the formation of thiocyanato derivatives, showing thereby that the yield of thiocyanato derivatives is not quantitative.

Scheit (1968) showed that in the oxidation of s^4U in 4-thiouridylyl-(3′-5′)-4-thiouridine with H_2O_2, the intermediate oxidation product is highly reactive and only uridine can be detected as the final product in the dinucleotide. Shugart (1972) also reported that there was no detectable s^4U in the unfractionated *E. coli* tRNA after 90min of exposure to H_2O_2. Our results show that H_2O_2 reacts with 92% of the sulphur in tRNA and the nucleosides affected are s^4U, mnm^5s^2U and ms^2i^6A. It is noteworthy that the s^2C peak is not affected by H_2O_2. However, it cannot be ruled out that an oxidative product of ms^2i^6A, as in $KMnO_4$ oxidation (see below) might be eluted in the place of s^2C.

Iida *et al.* (1973) have described the reaction of NH_2OH with s^4U monomer in neutral aqueous solutions to give N^4-hydroxycytidine. The reaction was supposed to involve the nucleophilic attack of hydroxylamine-free base towards C-4 of the undissociated s^4U. Our experiments show that NH_2OH reacts with all the four thio-bases present in tRNA to various extents. Under conditions where s^4U as the monomer would be almost completely desulphurized, ^{35}S-labelled tRNA loses only 20% of its sulphur. On conversion into nucleosides involving alkali treatment, the NH_2OH-treated tRNA loses 56% more of its sulphur than does the control. This could not be possible if the desulphurization takes place solely by the nucleophilic attack of hydroxylamine. The loss of sulphur by NH_2OH-treated tRNA on alkali treatment after the removal of NH_2OH from tRNA indicates prior labilization of sulphur in the tRNA by NH_2OH. Hydroxylamine is known to possess oxidative properties (Martz & Quin, 1969); thus the thio-bases may also be oxidized by NH_2OH in addition to the nucleophilic removal of sulphur, as suggested by the two-step removal of sulphur from tRNA.

Iodine oxidation of tRNA was the earliest chemical modification attempted on thionucleosides in tRNA. In fact, the clue to the presence of thio-bases in tRNA was provided by the susceptibility of *E. coli* tRNA to oxidation with iodine (Carbon *et al.*, 1965). Lipsett & Doctor (1967) showed for the first time the formation of intramolecular disulphide between two s^4U molecules in *E. coli* $tRNA^{Tyr}$ on oxidation with iodine without loss of acceptor activity. Therefore the observed loss of acceptor activity of certain species of tRNA on iodine oxidation was ascribed to the formation of disulphide bonds between 2-thiopyrimidines present in tRNA (Carbon & David, 1968). But

the thio-base composition of various species of *E. coli* tRNA shows that apart from the already known $tRNA^{Tyr}$ only $tRNA^{Lys}$ contains two s^4U residues, and there is no indication of other species containing more than one thiopyrimidine residue (Nishimura, 1972). Moreover, the results presented here and elsewhere (Faulkner & Uziel, 1971) rule out the formation of intermolecular disulphide bonds. Thus we consider that the cause for the observed loss of amino acid-acceptor activity of tRNA on iodine oxidation of tRNA should be sought elsewhere than dimer formation. It is shown here that reaction with iodine results in the desulphurization of tRNA by up to 25% and it also labilizes another 20% of the sulphur in tRNA. Thus the inhibition of acceptor activity of tRNA by iodine may be related to the desulphurization phenomenon observed here. In this context it is noteworthy that $tRNA^{Lys}$, most affected by iodine oxidation (Carbon *et al.*, 1965), is also most affected when desulphurized by CNBr, as shown here. Gross & Czerny (1973) reported the temperature-dependent inactivation of *E. coli* $tRNA^{Tyr}$ acceptor function by iodine oxidation, independent of dimer formation. They also observed that iodine treatment caused the loss of a positive charge of the nucleoside Q. The work of Faulkner & Uziel (1971) showed that on iodine treatment ms^2i^6A forms various products of yet unknown structure, and ^{131}I could be detected in ms^2i^6A. Lipsett (1967) reported that iodine oxidation of *E. coli* tRNA resulted in the quantitative conversion of s^4U into its dimer. As discussed above, the intermolecular dimer formation is ruled out and only two tRNA species contain more than one s^4U residue in *E. coli*. Moreover our results show that a maximum of only 6% of the total sulphur was involved in dimer formation on iodine oxidation. The reported observation of quantitative conversion of s^4U into its dimer on iodine oxidation of tRNA might be a secondary reaction observed due to oxidation of the 4-thiouridylate monomer after the hydrolysis of tRNA by any residual iodine not completely removed after the reaction. Whether the 6% of sulphur involved in dimer formation comes from $tRNA^{Tyr}$ only or also from $tRNA^{Lys}$ is not clear.

HNO_2 has been widely used in biochemical studies of nucleic acids. A reaction of HNO_2 with s^4U resulting in the desulphurization of the latter was reported by Iida & Hayatsu (1971). This reaction was observed to take place more rapidly than the known deamination of nucleosides. It was also observed that the desulphurization of s^4U by HNO_2 involved the intermediate formation of bis-(4-thiouridine) disulphide, although it was suggested that the formation of the disulphide may not be a prerequisite to the desulphurization of s^4U. Our results indicate that mnm^5s^2U and s^2C are also susceptible to HNO_2 treatment, as well as s^4U. We do not know how much of the s^4U in tRNA was desulphurized via dimer formation on treatment with HNO_2, though it is a known intermediate in the desulphurization of s^4U as the monomer. Such an intermediate, if it were to form, would involve intermolecular disulphide formation in tRNA, as it is known that most of the tRNA species in *E. coli* contain only one thiopyrimidine residue in their primary structure (see above).

The studies by Yano & Hayatsu (1970) on permanganate oxidation of nucleosides have shown that pyrimidine bases but not purine bases can be oxidized rapidly by treatment with dilute $KMnO_4$. Of the pyrimidines tested s^4U was found to be most susceptible to the oxidation. Thus s^4U could be selectively oxidized with $KMnO_4$ to form uridine 4-sulphonate, which is very reactive to nucleophilic attack. Under similar conditions of oxidation leading to sulphonate formation, we observed some 15% loss of the sulphur from tRNA. Moreover, only 50% of the sulphur in tRNA was oxidized by $KMnO_4$ under conditions when s^4U would have been oxidized completely as the monomer, showing that s^4U in tRNA is slow to react with $KMnO_4$. Under the conditions used here, only thymidine among the major nucleosides is known to be affected by $KMnO_4$ by up to 8% as the nucleoside (Yano & Hayatsu, 1970). It is not known here how much of the ribothymidine in tRNA is affected by $KMnO_4$. Hall (1970) reported that treatment of N^6-isopentenyladenosine with $KMnO_4$ resulted in the formation of a dihydroxylated derivative and adenosine. If the same reaction is assumed to take place with ms^2i^6A, it may be conjectured that the dihydroxylated derivative is the new peak that was eluted between s^4U and guanosine, and the 2-methylthioadenosine is that eluted in the position of s^2C, thus increasing its apparent relative proportion (Fig. 5*c*). Also the fate of s^2C on $KMnO_4$ oxidation could not be ascertained owing to the interference of the ms^2i^6A derivative.

Bisulphite has been used to convert specifically the cytidine residues in the non-helical regions of tRNA (Singhal, 1971; Kucan *et al.*, 1971). Under similar conditions we observed here that s^4U, mnm^5s^2U and s^2C were affected by bisulphite whereas no reaction could be observed with ms^2i^6A. Thus these results provide a note of caution that those who use bisulphite for the modification of tRNA have to take note of the reactivity of these minor bases as well. As for the reaction of bases with bisulphite, several interactions are known depending on the reaction conditions. Bisulphite adds to the 5–6-double bond of pyrimidine nucleosides (Hayatsu *et al.*, 1970*a*,*b*; Shapiro *et al.*, 1970). It was also found to add to the isopentenyl moiety of the N^6-isopentenyladenosine in tRNA (Furuichi *et al.*, 1970). The O_2-dependent reaction of s^4U with bisulphite, resulting in the formation of uridine 4-sulphonate, was investigated by Hayatsu & Inoue (1971). The formation of uridine 4-sulphonate is mediated by uridine 4-thiosulphate (a 'Bunte salt')

and the sulphur in the product, uridine 4-sulphonate, was found to be from the bisulphite of the medium. Also hydroquinone and high concentrations of bisulphite were found to inhibit the formation of uridine 4-sulphonate, showing the involvement of free radicals in the reaction. As the conditions used by Hayatsu & Inoue (1971) (10mM-bisulphite, O_2 bubbling at pH7.0) were different from those used here (2M-bisulphite, pH5.8), the mechanism of desulphurization may be different.

The reaction of s^4U as the monomer with $NaIO_4$ is well documented (Ziff & Fresco, 1968). Although the tRNA treated with $NaIO_4$ has been used as substrate for the tRNA sulphur transferase enzyme, the extent of desulphurization has not been established. Moreover, there are conflicting reports as to the suitability of tRNA desulphurized with $NaIO_4$ as substrate for tRNA sulphur transferase (Hayward & Weiss, 1966; Lipsett & Peterkofsky, 1966). We find that periodate removes sulphur almost completely from tRNA. A method for the purification of amino acid-specific tRNA species involving modification with periodate has been reported (Stephenson & Zamecnik, 1967). The conditions required for such modification are essentially the same as those used by us to modify tRNA. Thus such procedures may be limited to tRNA species that do not contain susceptible bases such as thio-bases. Other sites of attack by periodate on the tRNA molecule include the *cis*-glycol of the 3'-nucleoside terminus (Whitfield & Markham, 1953).

The role of the minor bases in tRNA is still not clear, though much work has been done towards its elucidation (Saneyoshi & Nishimura, 1971). An attempt has been made here to understand the role of thionucleosides in the recognition of aminoacyl-tRNA synthetase by removing sulphur completely from the thiopyrimidines in tRNA by CNBr treatment and determining the amino acid-acceptor activity. From the results obtained, it is noteworthy that the three tRNA species, namely $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Lys}$, that are most affected by desulphurization contain mnm^5s^2U in the first position of the anticodon. It seems possible that the first letter of the anticodon or the anticodon loop of these three tRNA species might be important for the recognition of aminoacyl-tRNA synthetase. The involvement of the anticodon region in the recognition of aminoacyl-tRNA synthetase has been suggested by several workers (Mirua, 1967; Carbon & Curry, 1968; Thiebe & Zachau, 1968; Mirzabekov *et al.*, 1971). However, there is also evidence that the anticodon loop is not part of the recognition site for aminoacyl-tRNA synthetase (Goodman *et al.*, 1968; Sundharadas *et al.*, 1968; Yoshida *et al.*, 1968; Imura *et al.*, 1969). The tRNA species corresponding to aspartic acid, asparagine and histidine, which contain the CNBr-susceptible Q base (Dirheimer *et al.*, 1972; Nishimura, 1972) in the first position of anticodon, are not affected, thus supporting the latter hypothesis. However, the tRNA for tyrosine, containing Q and s^4U, lost about 60% of the activity. Therefore the recognition site for aminoacyl-tRNA synthetase in the tRNA molecule may differ for different tRNA species (Chambers, 1971). The acceptor activities of arginine, glycine, methionine, phenylalanine, tyrosine, valine and serine are affected by up to 50%, owing to desulphurization, showing thereby the involvement of sulphur in aminoacyl-tRNA synthetase recognition. These results broadly agree with those reported by Saneyoshi & Nishimura (1971) and Harris *et al.* (1969), but differ in detail. The former authors prepared thiocyanato derivatives of *E. coli* tRNA and determined the amino acid-acceptor activity, whereas in the present studies sulphur was completely removed from the thiopyrimidines by incubation of the tRNA containing the thiocyanato derivatives at pH4.7. Harris *et al.* (1969) observed decreased amino acid acceptance in preparations of partially sulphur-deficient tRNA species isolated from 'relaxed' cysteine auxotrophs of *E. coli*. However, Walker & RajBhandary (1972) and Doctor *et al.* (1969), who worked with purified species of tyrosine tRNA and formylmethionine tRNA, reached the conclusion that s^4U is not essential for the acceptor activities of these tRNA species.

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References

Burrows, W. J., Armstrong, D. J., Skoog, F., Hecht, S. M., Boyle, J. T. A., Leonard, N. J. & Occolowitz, J. (1968) *Science* **161**, 691–693

Carbon, J. & Curry, J. B. (1968) *J. Mol. Biol.* **38**, 201–216

Carbon, J. A. & David, H. (1968) *Biochemistry* **7**, 3851–3858

Carbon, J. A., Hung, L. & Jones, D. S. (1965) *Proc. Nat. Acad. Sci. U.S.* **53**, 979–986

Carbon, J. A., David, H. & Studier, M. H. (1968) *Science* **161**, 1146–1147

Chambers, R. W. (1971) *Progr. Nucl. Acid Res. Mol. Biol.* **11**, 489–525

Cherayil, J. D., Hampel, A. & Bock, R. M. (1968) *Methods Enzymol.* **12**, 166–169

Dirheimer, G., Ebel, J. P., Bonnet, J., Gangloff, J., Keith, G., Krebs, B., Kuntzel, B., Roy, A., Weissenbach, J. & Werner, C. (1972) *Biochimie* **54**, 127–144

Doctor, B. P., Wayman, B. J., Cory, S., Rudland, P. S. & Clark, B. F. C. (1969) *Eur. J. Biochem.* **8**, 93–100

Faulkner, R. D. & Uziel, M. (1971) *Biochim. Biophys. Acta* **238**, 464–474

Furuichi, Y., Wataya, Y., Hayatsu, H. & Ukita, T. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1185–1191

Goodman, H. M., Abelson, J., Landy, A., Brenner, S. & Smith, J. D. (1968) *Nature (London)* **217**, 1019–1024

Gross, H. J. & Czerny, E. M. (1973) *FEBS Lett.* **30**, 347–350
Hall, R. H. (1970) *Progr. Nucl. Acid Res. Mol. Biol.* **10**, 57–86
Hampel, A., Cherayil, J. & Bock, R. M. (1971) *Biochim. Biophys. Acta* **228**, 482–492
Harris, C. L., Titchener, E. B. & Cline, A. L. (1969) *J. Bacteriol.* **100**, 1322–1327
Hayward, R. S. & Weiss, S. B. (1966) *Proc. Nat. Acad. Sci. U.S.* **55**, 1161–1168
Hayatsu, H. & Inoue, M. (1971) *J. Amer. Chem. Soc.* **93**, 2301–2306
Hayatsu, H., Wataya, Y. & Kai, K. (1970*a*) *J. Amer. Chem. Soc.* **92**, 724–726
Hayatsu, H., Wataya, Y., Kai, K. & Iida, S. (1970*b*) *Biochemistry* **9**, 2858–2865
Hecht, S. M., Kirkegaard, L. H. & Bock, R. M. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 48–51
Iida, A., Chung, K. C. & Hayatsu, H. (1973) *Biochim. Biophys. Acta* **308**, 198–204
Iida, S. & Hayatsu, H. (1971) *Biochem. Biophys. Res. Commun.* **43**, 163–167
Imura, N., Schwam, H. & Chambers, R. W. (1969) *Proc. Nat. Acad. Sci. U.S.* **62**, 1203–1209
Kochetkov, N. K. & Budowsky, E. I. (1969) *Progr. Nucl. Acid Res. Mol. Biol.* **7**, 403–438
Kucan, Z., Freude, K. A., Kucan, I. & Chambers, R. W. (1971) *Nature (London) New Biol.* **232**, 177–179
Lipsett, M. N. (1965) *J. Biol. Chem.* **240**, 3975–3978
Lipsett, M. N. (1967) *J. Biol. Chem.* **242**, 4067–4071
Lipsett, M. N. & Doctor, B. P. (1967) *J. Biol. Chem.* **242**, 4072–4077
Lipsett, M. N. & Peterkofsky, A. (1966) *Proc. Nat. Acad. Sci. U.S.* **55**, 1169–1174
Martz, M. D. & Quin, L. D. (1969) *J. Org. Chem.* **34**, 3195–3196
Mirua, K. (1967) *Progr. Nucl. Acid Res. Mol. Biol.* **6**, 39–82
Mirzabekov, A. D., Lastity, D., Levina, E. S. & Bayev, A. A. (1971) *Nature (London) New Biol.* **229**, 21–22
Muench, K. K. & Berg, P. (1966) *Proc. Nucl. Acid Res.* **1**, 375–383
Nishimura, S. (1972) *Progr. Nucl. Acid Res. Mol. Biol.* **12**, 49–85
Rao, Y. S. P. & Cherayil, J. D. (1973) *Biochim. Biophys. Acta.* **299**, 1–7
Saneyoshi, M. & Nishimura, S. (1967) *Biochim. Biophys. Acta* **145**, 208–210
Saneyoshi, M. & Nishimura, S. (1970) *Biochim. Biophys. Acta* **204**, 389–399
Saneyoshi, M. & Nishimura, S. (1971) *Biochim. Biophys. Acta* **246**, 123–131
Saneyoshi, M., Anami, T., Nishimura, S. & Samejima, T. (1972) *Arch. Biochem. Biophys.* **152**, 677–684
Scheit, K. H. (1968) *Biochim. Biophys. Acta* **166**, 285–293
Shugart, L. (1972) *Arch. Biochem. Biophys.* **148**, 488–495
Shapiro, R., Servis, R. E. & Welcher, M. (1970) *J. Amer. Chem. Soc.* **92**, 422–424
Singhal, R. P. (1971) *J. Biol. Chem.* **246**, 5848–5851
Stephenson, M. L. & Zamecnik, P. C. (1967) *Methods Enzymol.* **12**, 670–678
Sundharadas, G., Katze, J. R., Soll, D., Konigsberg, W. & Lengyel, P. (1968) *Proc. Nat. Acad. Sci. U.S.* **61**, 693–700
Thiebe, R. & Zachau, H. G. (1968) *Biochem. Biophys. Res. Commun.* **33**, 260–265
Uziel, M. (1966) *Biochem. Biophys. Res. Commun.* **25**, 105–108
von Ehrenstein, G. (1967) *Methods Enzymol.* **12**, 588–596
Walker, R. T. & RajBhandary, U. L. (1970) *Biochem. Biophys. Res. Commun.* **38**, 907–914
Walker, R. T. & RajBhandary, U. L. (1972) *J. Biol. Chem.* **247**, 4879–4892
Whitfield, P. R. & Markham, R. (1953) *Nature (London)* **171**, 1151–1152
Yaniv, M., Favre, A. & Barrell, B. G. (1969) *Nature (London)* **223**, 1331–1333
Yano, M. & Hayatsu, H. (1970) *Biochim. Biophys. Acta* **199**, 303–315
Yoshida, M., Furuichi, Y., Kaziro, Y. & Ukita, T. (1968) *Biochim. Biophys. Acta* **166**, 636–645
Ziff, E. B. & Fresco, J. R. (1968) *J. Amer. Chem. Soc.* **90**, 7338–7342