METHIONINE TRANSFER RNA FROM AN

EXTREMELY HALOPHILIC BACTERIUM

METHIONINE TRANSFER RNA FROM AN EXTREMELY HALOPHILIC BACTERIUM

by

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This thesis proposed to investigate the role played by methionine transfer RNA in the initiation of protein synthesis in the extreme halophile Halobacterium cutirubrum. Techniques were demonstrated for the isolation of two isoaccepting $tRNA^{met}$ species. One of these species, the late eluting $tRNA^{met}$ on BD-cellulose and RPC-5, was tentatively identified as a non-formylated initiator.

Acknowledgements

I would like to thank my supervisor, Dr. S.T.Bayley for his help and patience throughout the course of this work. I am especially indebted to my typist, Mrs. M.Hobart, both for generosity and her ability. Finally, credit must be given to all the members of the Grand Academy of Lagado.

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I INTRODUCTION

1 Translational Initiation

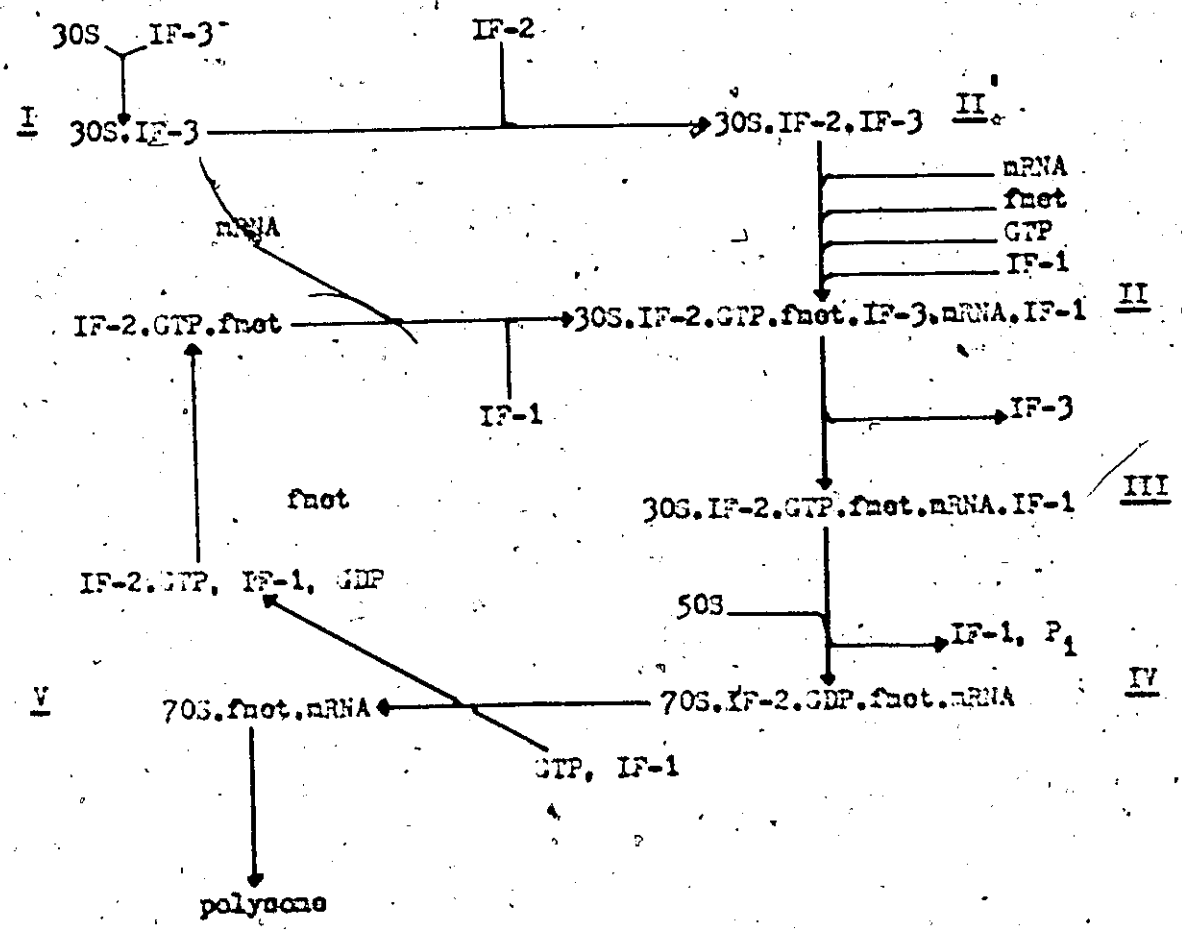
Significant advances dealing with initiation of protein synthesis first appeared in the literature in 1966. Working with Escherichia coli, Clark & Marcker; Adams and Capecchi; Webster, Engelhardt and Zinder; all independently implicated methionine as the N-terminal amino acid, with introduction into protein mediated through N-formyl-methionine. These early results gave rise to a very rapid progress and the components and mechanism of translational initiation are now fairly well understood (Lengyel & Söll, 1969; Bretscher, 1971). Although the exact sequence of events and the detailed interactions are to date still unknown an overall pathway may be adequately represented as in figure 1.

This scheme illustrates what has come to be regarded as prokaryotic initiation; this includes mitochondria and chloroplasts and is characterised by the presence of f-met-tRNA$_f^{met}$ and 70S ribosomes (Marcker & Smith, 1969). The latter characterisation may not be universally applicable since the use of non-formylated met-tRNA has been implicated in E.coli infected with a T_4 phage mutant unable to synthesise tetrahydrofolate (Klein et al. 1968), a non folate requiring mutant of Streptococcus faecalis (Samuel et al. 1972), and H.cutirubrum (White & Bayley, 1972c), but nevertheless it still serves at present to indicate a fundamental difference from the process of eukaryotic initiation. The latter form of initiation, although thought to proceed in a similar manner, is presently characterised by the use of non-formylated met-tRNA^{met} and 80S ribosomes (Marcker & Smith, 1969). The non-formylated initiator may however be formylated by an E.coli transformylase.

Figure 1

Prokaryotic initiation

from Haselkorn & Rothman-Denes (1973).



IF-1,2,3 = initiation factors.

fmet = formylmethionine $tRNA_f$.

I - V = key stages in the process.

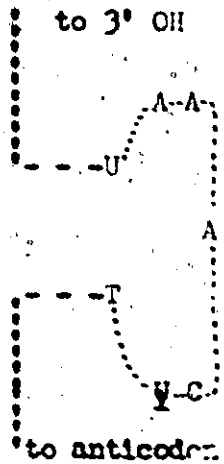
IF-1 is probably needed only for the release of IF-2 in step IV ⟶ V, but it has been observed to associate with complexes like II and III in vitro, so it is included there as well.

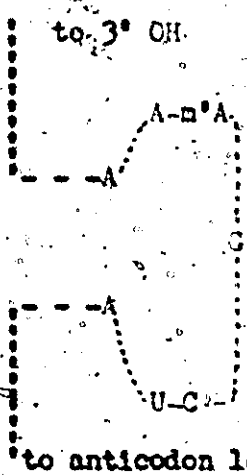
as in the case of yeast, mouse liver, rat liver and rabbit reticulocytes (Smith & Marcker, 1970), or it may not be recognised by the E.coli transformylase as in the case of wheat embryo (Ghosh et al. 1971).

The requirement for the presence of a formyl group was never fully understood. In vivo studies with E.coli treated with trimethoprim, which blocks the synthesis of tetrahydrofolate, the formyl group donor, did not result in complete inhibition of protein synthesis (Bretscher, 1971) and in vitro assays with E.coli did not require formylation for initiation activity (Clark & Marcker, 1966). The conclusion drawn by the latter authors called for a structural specificity of the initiator tRNA and the presence of the formyl group to prevent that tRNA from internal insertion of methionine and also to enhance the rate of initiation. The discovery of non-formylated initiators intensified research, both in terms of the isolation and structural analyses of the $tRNA^{met}$ species and the macromolecular interactions necessary for terminal and internal insertion.

The in vitro results reported thus far (Chatterjee et al. 1971; Drews, Grasmuk and Weil, 1972; Ghosh and Ghosh, 1972) have all strengthened the views of Clark and Marcker. Using physiological assay conditions, i.e. low Mg^{2+}, specificity both for $tRNA^{met}$ structure and the formyl group has been demonstrated with initiation and elongation factors. It is now believed that with eukaryotic systems the formyl group requirement has been eliminated by virtue of structural changes in the tRNA and in the initiation and elongation factors, resulting in a much stricter specificity of interaction. The nature of the structural changes in the initiator $tRNA^{met}$ has recently been proposed by Simsek et al. (1972, 1973) to

reside in a unique nucleotide sequence lacking a pseudouracil base:-





E.coli $tRNA_f^{met}$ - Dube et al. 1968 presence of pseudouracil at this position universal to all tRNAs active in protein synthesis which have so far been sequenced.

unique sequence in rabbit liver, sheep mammary gland, yeast, wheat embryo } Initiator $tRNA^{met}$

The initiator $tRNA^{met}$ species studied thus far have all possessed certain properties concomitant with their specialised function. Principally amongst these has been their ability to initiate protein synthesis in vitro at low magnesium concentrations (below 10mM) in the presence of initiation factors and mRNA containing an initiation codon (Lengyel & Söll, 1969). The occurrence of two such codons AUG and GUG (Ghosh et al. 1967; Stoltz, 1969) highlights another unique property. This type of degeneracy towards the first base in the codon is opposite to the "wobble" hypothesis of Crick (1966) and is not shown by any other tRNA. The initiator $tRNA^{met}$ also possesses the ability to bind directly to ribosomes in response to its appropriate triplets in such a fashion that it can react immediately with puromycin, resulting in the formation of methionyl-puromycin (Bretscher &

Marcker, 1966). This ability is not shown by any other aminoacyl-tRNA, including the propagator met-tRNA^{met}, and as such has provided *in vitro* an assay specific for the initial peptide bond (Leder & Bursztyn, 1966), and *in vivo* an experimental approach suitable for detection of the initial amino acid (Bachmayer & Kreil, 1968).

The distinctive features of the initiator tRNA^{met} species as described above should serve to determine which, if either, of the two methionine accepting species of tRNA present in *H.cutirubrum* (White & Bayley, 1972c) could serve as an initiator tRNA.

2. Halophilism

The bacteria to which this thesis relates is unusual in a number of respects and therefore it seems appropriate at this point to review some of its characteristic features so that the later results can be placed in their proper context. This introduction will of necessity be very limited and reference must therefore be made to the complete reviews provided by Larsen (1967) and Kushner (1968).

Halobacterium cutirubrum is an extreme halophile, as defined by its requirement for 20 - 30% NaCl in the medium for optimal growth (Larsen, 1962). It is a member of the family Pseudomonadaceae, as described in the 7th edition of Bergey's Manual (Breed, Murray & Smith, 1957), and is one of five species which together comprise the genus Halobacteria. These bacteria are slender rods, 1 x 6 micron, which are obligate aerobes and are pigmented with carotenoids. They are gram negative, do not form spores and possess polar flagella when motile. They require at least 12 - 15% NaCl for growth and they undergo lysis when subjected to low salt concentration, 5% NaCl. A study by Moore & McCarthy (1969a) showed that strains of extremely halophilic rods were closely related but they were not related to the genus Halococcii, the only other extreme halophiles, or to the less halophilic bacteria.

The biochemical features which at present are used to characterise the Halobacteria are directed towards their macromolecules. The DNA's of these bacteria possess a high GC content, 67%, and they all contain satellite DNA of GC content 57 - 60%, whose function is unknown but whose extent varies between 10 - 36% of the total DNA depending on the species (Moore & McCarthy, 1969b). They possess unique phospholipids which are

derivatives of glycerol diether rather than the usual diester (Kates, 1965, 1966), and the cellular envelope appears to be composed mainly of lipoprotein lacking a rigid mucopeptide layer (Brown & Shorey, 1963; Brown, 1964; Kushner, 1964). The structural proteins studied thus far appear to be acidic in nature, as shown by the ribosomal proteins (Bayley & Kushner, 1964; Bayley, 1966) and the envelope proteins (Brown, 1963; Kushner, 1964; Kushner and Onichi, 1966). Finally, in many cases the enzymes themselves are halophilic in their salt requirements for both stability and activity (Baxter, 1959; Holmes & Halvorson, 1963). The features listed above probably represent the major adaptations of the Halobacteria but as such they should be considered secondary to the fundamental characteristic, namely that these bacteria grow, and indeed can only survive in nearly saturated brine.

The adaptation towards high salt has not been simply a consequence of the development of an unusual permeability barrier since it has been shown for H.salinarium that the intracellular monovalent cation concentration is substantially greater than that of the external medium (Christian & Waltho, 1962). These authors also indicated that the membrane possessed a selectivity for the concentration of potassium ion and the exclusion of sodium; the figures quoted were for an external medium of 4.0M NaCl and 0.03M KCl and intracellular concentrations of 4.6M KCl and 1.4M NaCl. It is clear therefore that the cellular machinery of the Halobacteria must function in a most hostile environment, one which is supersaturated in monovalent cation.

Investigations into the phenomenum of halophilism have been attempted in the hope of achieving two main goals. Firstly, as a means of understanding the adaptation and evolution of the biological processes towards

a high salt environment and secondly, as a means of gaining an insight into the fundamental nature of the processes themselves. In these respects the mechanism of protein synthesis in H.cutirubrum has been examined in some detail.

The cell-free systems produced by Bayley & Griffiths (1968a,b) and Griffiths & Bayley (1969) were all truly halophilic in their requirement for nearly saturating salt conditions. However, the components and reactions involved were shown to be basically similar to those from non-halophilic organisms and this even extended to specific cationic requirements, notably Mg^{2+} and NH_4^+. On the basis of these results the authors concluded that the essential features of translation viz. codon-anticodon recognition, aminoacyl-tRNA formation and transfer of aminoacyl-tRNA had remained unchanged. Later work reinforced this conclusion and it became more apparent that the nucleic acid components of protein synthesis had not been drastically modified.

The ribosomes, although possessing mainly acidic proteins and requiring high salt conditions, were found to be similar in size to those of non-halophilic bacteria and to require similar Mg^{2+} concentrations for the association of 50S and 30S subunits (Bayley, 1966; Bauser & Bayley, 1968). The response of the cell-free systems towards synthetic templates showed that the genetic code was essentially unaltered (Bayley & Griffiths, 1968b; White & Bayley, 1972a). Finally, the halophilic tRNA was shown not to possess fundamental structural differences from E.coli tRNA as evidenced by the heterologous interactions reported by White & Bayley (1972b).

Only one main difference thus far, apart from the salt conditions, has been reported at variance with other prokaryotic systems and this con-

cerned the process of initiation. Methionine, by virtue of its stimulated incorporation with poly (GU) (Bayley & Griffiths, 1968b), had been implicated in an initiating role, but no evidence was found for the presence of formylmethionine or a formylating system (White & Bayley, 1972c). However, the results indicated that one species of $tRNA^{met}$ could be formylated in an E.coli system, and therefore it was considered possible that initiation in this prokaryotic halophilic system was similar to that believed to occur in eukaryotic organisms.

This thesis was designed to investigate the possible involvement of $tRNA^{met}$ in the initiation of protein synthesis in H.cutirubrum.

II. METHODS.

1 Growth of Bacteria

The studies reported in this thesis pertained to Halobacterium cutirubrum strain 9, obtained from the National Research Council in Ottawa.

The cells were grown in the medium of Sehgal and Gibbons (1960), with the exceptions that 10 ppm Fe^{2+} (as $FeSO_4$) were added and the final pH was 6.2. The principal inorganic salts in this complex medium were 4.3M NaCl, 0.27M KCl, and 0.08M $MgSO_4$; its exact composition and preparation are described in table 1.

Batch cultures (12L.) were grown in a 14L. New Brunswick Microfern bench fermentor at 37°C under continuous aeration, air flow 3L. per minute, with a stirring speed of 300 rpm. Foaming was prevented by the addition of Dow Corning antifoam A reagent. The cultures were inoculated with a 5% starting inoculum grown for 24 hrs. in an Erlenmeyer flask in a rotary shaker.

Harvesting was achieved at 0 - 4°C by centrifugation at 7000 rpm for 10 min. in the Sorvall GSA rotor, or preferably in a Sharples continuous centrifuge running at 30,000 - 35,000 rpm. The cells were washed twice with centrifuging in a solution containing 4.3M NaCl, 0.03M KCl, and 0.08M $MgSO_4$ and were either used directly for cell-free extracts or frozen and stored at -40°C for later preparation of transfer RNA.

The growth of H.cutirubrum under the conditions described is shown in figure 2.

Table 1

Complex liquid medium for culturing H.cutirubrum

Yeast extract	(Difco)	10 g.
Casamino acids	(Difco)	7.5g.
KCl		2 g.
$MgSO_4.7H_2O$		20 g.
NaCl		250 g.
Sodium citrate		3 g.
$FeSO_4$		10ppm.

Volume adjusted to 1 litre with distilled water

pH adjusted to 7 - 8 with NaOH

Autoclaved 5 min.

Volume of filtrate adjusted to 1 litre

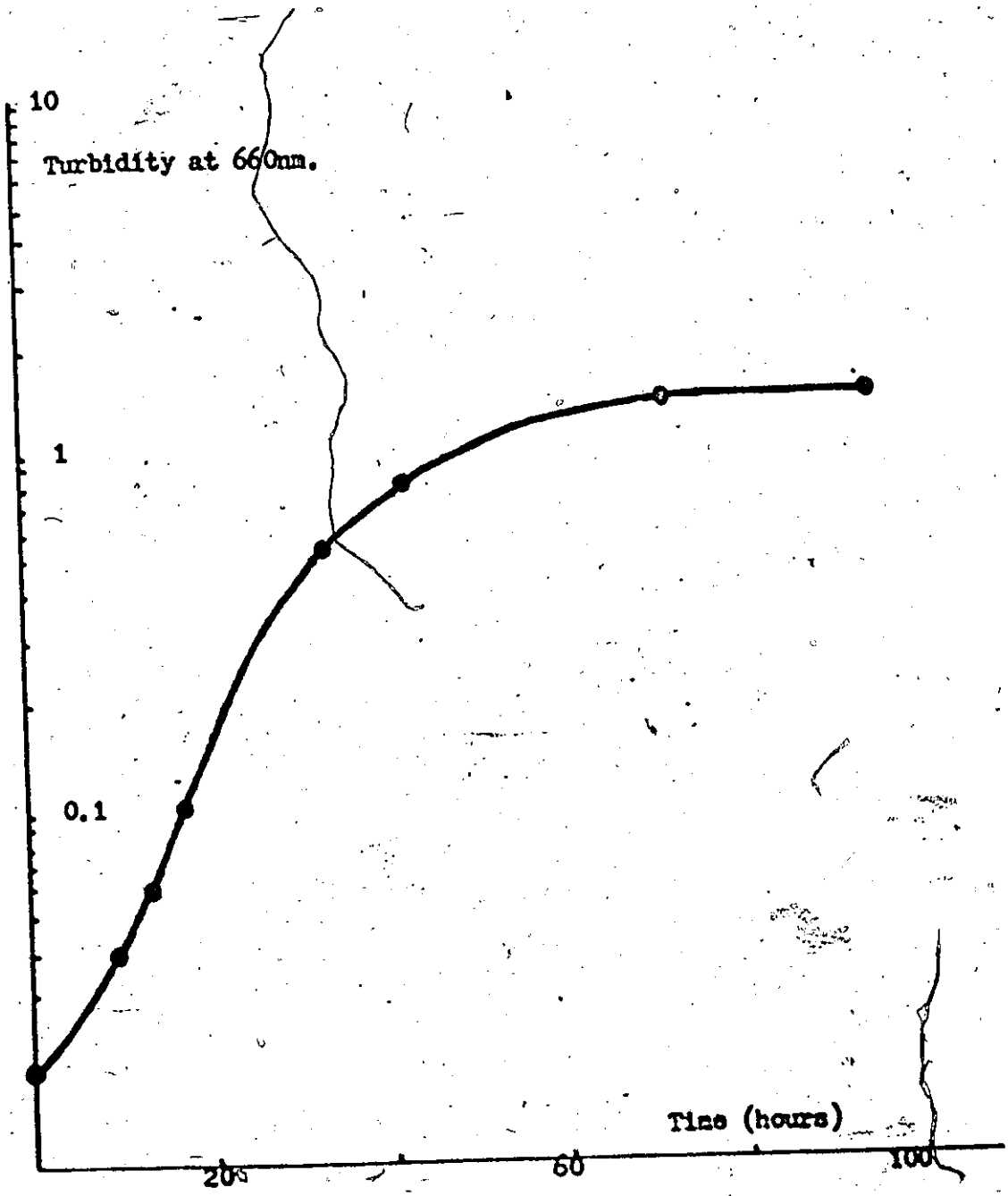
pH adjusted to 6.2 with HCl

Autoclaved 20 min.

Figure 2

Growth of H.cutirubrum in a New Brunswick Fermentor

12 litres of complex medium in a 14L. vessel were inoculated with 600ml. of a 24hr. shake culture. Optical density readings were taken with a Bausch and Lomb Spectronic 20 at 660nm.


10
Turbidity at 660nm.
1
0.1
Time (hours)
20
60
100

2 Preparation of biological extracts

A H.CUTIRUBRUM

All procedures were at 0 - 4°C. unless otherwise stated.

Solution D' contained 3.0M KCl, 0.1M Tris-HCl, pH 7.6, 0.1M MgAc and 1mM 2-mercaptoethanol.

i S-60 extract ;Bayley & Griffith (1968a)

Harvested, washed cells were homogenised in a glass Teflon Potter-Elvenhjem homogeniser with a volume of solution D' corresponding to 1 - 1.5 times their wet weight, together with 1mg. of electrophoretically purified DNAse per 30ml. of added D'. The DNAse was added as a solution of 1mg./ml. in 0.1M potassium acetate, pH 5.0.

The crude S-60 extract was obtained from the homogenate by centrifugation, once at 40,000g. for 20min. and twice at 60,000g. for 30min.; in each case only the upper $^4/_5$ of the supernatant was retained. The extract was thoroughly dialysed against solution D' and then was either used directly for further preparations or frozen in vials in isopentane at liquid nitrogen temperature and stored under liquid N_2.

ii S-150 extract ;Bayley & Griffith (1968b)

S-150 extracts were obtained by centrifugation of the S-60 supernatant at 150,000g. for 2.5hrs. The upper $^4/_5$ of the resultant supernatant was then frozen and stored under liquid N_2.

iii Synthetase preparation

Separation of transfer RNA and aminoacyl-synthetase was achieved by either pH 5.0 treatment of the S-150 according to Griffith and Bayley (1969) or by gel filtration of the S-150 or its 100% $(NH_4)_2SO_4$ precipitate on a Sephadex G-50 or G-75 column in solution D' according to B.N.White (1970). In both cases the synthetase fraction was frozen and stored in solution D' under liquid N_2.

The same Sephadex procedure also provided a source of transferases but this was contaminated with considerable amounts of synthetases (B.N.White, 1970).

iv Ribosomes ; Bayley & Griffith (1968b)

Ribosomes free of endogenous mRNA activity were prepared by pre-incubation of S-60 extracts in bulk at 37°C for 40min. in the reaction mixture described in table 2. The ribosomes were isolated from this mixture by centrifugation at 150,000g. for 2.5hrs. The resultant pellet was suspended in solution D' using a glass Teflon Potter-Elvenhjem homogeniser and again sedimented at 150,000g. for 2hrs. The pellet was again suspended in solution D', clarified by low speed centrifugation and frozen and stored under liquid N_2.

Table 2

Reaction mixture for bulk incubation of S-60 extracts in the preparation of ribosomes free of endogenous mRNA

Na ATP (0.03M)	0.25 ml.
Na PEP (0.12M)	0.25 ml.
Na GTP (0.012M)	0.375 ml.
1M Tris-HCl, pH 8.05	0.125 ml.
5M NH_4Cl + 0.6M $(NH_4)_2SO_4$	0.75 ml.
Solid KCl	0.615 g.
S-60 extract in D'	2.00 ml.
total nominal volume *	3.75 ml.

nominal final conditions:

3.8M KCl, 1.2M NH_4^+, 0.05M MgAc, 2mM ATP, 8mM PEP, 0.8mM GTP.

* no allowance has been made for the volume of dry salt added and therefore the final concentrations are slightly lower than those provided above.

v Transfer RNA

Two methods were employed :-

a - according to Griffiths & Bayley (1969)

Frozen or freshly harvested cells were homogenised, together with a volume of D' equal to their wet weight, in a Sorvall omnimixer at top speed for 30sec. The homogenate was incubated with DNAse, 1mg./30ml. D', for 30min. at 37°C before being centrifuged to give an S-150 supernatant.

The pH of the supernatant, after pH 5.0 removal of synthetases was readjusted to 7.6 with 1M KOH and the salt concentration was reduced by overnight dialysis against 5mM MgAc, Tris-HCl, pH 7.6, 2-mercaptoethanol. After dialysis, 0.01 volume of sodium dodecyl sulphate, 0.125mg./ml., was added and the solution stirred thoroughly for 20min. Transfer RNA was then isolated by the usual phenol extraction procedure and precipitated with potassium acetate, pH 5.0, 20% $^{w}/_{v}$, and ethanol (Kirby, 1956; von Ehrenstein and Lipmann, 1961; Noldave, 1963). The precipitated tRNA was redissolved in the low salt buffer and reprecipitated with potassium acetate as before.

The final precipitate was then successively washed with ethanol-water (2:1), ether-ethanol (1:2, 1:1, 2:1) and finally anhydrous ether before being dried under vacuum and stored as the dry powder at -20°C (preparation A). Alternatively, the final precipitate was dissolved in distilled water and dialysed extensively against 2mM 2-mercaptoethanol.

The tRNA was then freeze-dried and the resultant dry powder stored at -20°C (preparation B).

b - according to Gunther von Ehrenstein (1968)

Frozen or freshly harvested cells were homogenised together with a volume of 0.1M NaCl, 0.01M $MgCl_2$, 0.01M Tris-HCl, pH 7.5 and 1mM 2-mercaptoethanol corresponding to 10x their wet weight and an equal volume of 88% phenol in a Waring blendor at top speed for one minute. The phases were separated by centrifugation and the lower phenolic phase was re-extracted with the above aqueous buffer. The nucleic acid was precipitated from the pooled aqueous phases by the addition of 2 volumes of 95% ethanol.

The high M.W. RNA and DNA were removed by means of an isopropanol fractionation (Zubay, 1962). The total nucleic acid precipitate was dissolved in 0.3M sodium acetate, pH 7.0, to which was then slowly added, dropwise from a separatory funnel, 0.54 volume of isopropanol. The temperature of the final solution was raised to 20°C and the high molecular weight material was sedimented by centrifugation. The supernatant was retained and the precipitated material was redissolved in 0.3M sodium acetate and reprecipitated as described above. The 2 supernatants were combined and tRNA was precipitated by the further addition of isopropanol to a final concentration of 0.9S volume.

The tRNA was then treated as in method a. either by washing through to ether and vacuum drying or by extensive dialysis against 1mM NaCl, 5mM 2-mercaptoethanol and freeze-drying. The dry powder was stored at -20°C.

B E.COLI

1 S-150

S-30 extracts, prepared according to Nirenberg (1964), were centrifuged at 150,000g. for 2.5hrs. The resultant supernatant (S-150) was dialysed against 0.03M KCl, 0.01M MgAc, 0.04M Tris-HCl, pH 7.8, and 6mM 2-mercaptoethanol and then frozen and stored under liquid N_2.

11 Washed ribosomes

The ribosomal pellet obtained by centrifugation of an S-30 extract at 150,000g. for 2.5hrs. was treated according to Lodar & Kurnstyn (1966a). The pellet was resuspended in 0.06M KCl, 0.01M MgAc, 0.01M Tris-HCl, pH 7.8 and 6mM 2-mercaptoethanol (buffer A) plus 0.5M NH_4Cl and dialysed against this solution for 2hrs. at 0°C. The ribosomes were recovered by centrifugation at 150,000g. for 2hrs. and resuspended in buffer A. They were again sedimented at 150,000g. for 2hrs. resuspended in buffer A, clarified by low speed centrifugation and frozen and stored under liquid N_2.

111 Transferase preparation

Transferases were obtained by $(NH_4)_2SO_4$ precipitation of the S-150 as described by Revel (1967).

3 In vitro assay systems

A H.CUTIRUBRUM

1 Polypeptide synthesis ;Bayley & Griffiths (1968a,b).

The reaction mixture is shown in table 3.

After 40 min. at 37°C the reaction tube was cooled to 0°C and to it was added 1000 fold excess of ^{12}C-amino acid corresponding to the ^{14}C-amino acid used, followed by 2ml. of cold aqueous 10% TCA. (If necessary 0.1ml. bovine serum albumin, 5mg./ml., was added as carrier.)

After 10min, at 0°C the precipitate formed was sedimented by centrifugation. The precipitate was washed twice with 5% TCA with centrifuging, heated to 90°C for 15 min. in 5% TCA (Siekevitz, 1952) cooled to 0°C for 15min. collected on a 0.45 micron Millipore filter and washed with 5% TCA (Nirenberg, 1964). The filter was dried at 60°C for 30 min. and then counted in a Beckman scintillation counter using omniflucr (4g./litre of toluene) as the scintillation fluid.

ii Aminoacyl-tRNA formation ;Griffiths & Bayley (1969).

The reaction mixture is shown in table 4. This mixture was used for all amino acids except methionine, in which case the NaCl was omitted (D.H.White, 1970).

After 20 min. at 37°C the reaction was terminated and treated as for polypeptide synthesis except the heating at 90°C was omitted.

Table 3

Reaction mixture for H.cutirubrum polypeptide synthesis

Solid KCl	20 mg.
4M NaCl + 1.6M NH_4Cl	0.025 ml.
0.6M Tris-HCl, pH 8.05	0.005 ml.
0.03M Na ATP + 0.012M Na GTP	0.005 ml.
0.12M Na PEP	0.005 ml.
19 ^{12}C-amino acids - ^{14}C-amino acid (each 8×10^{-4}M)	0.005 ml.
^{14}C-amino acid (neutralised)	0.005 ml.
Polyribonucleotide (5mg./ml. H_2O)	0.010 ml.
Solution D' *	0.040 ml.
total nominal volume	0.100 ml.

* Addition of ribosomes, S-150, synthetases, transferases and tRNA as required

nominal final conditions:

3.8M KCl, 1M NaCl, 0.4M NH_4Cl, 0.03M Tris-HCl, pH 8.05, 1.5mM ATP

0.6mM GTP, 6mM PEP, 4mmoles of each ^{12}C-amino acid,

1 - 10mmoles ^{14}C-amino acid, 0.05mg. polyribonucleotide,

2 - 4 A_{260} units of ribosomes.

Table 4

Reaction mixture for H.cutirubrum aminoacylation

Solid KCl	43.61 mg.
5.6M NaCl	0.055 ml.
0.29M Tris-HCl, pH 8.0	0.020 ml.
0.3M Na ATP	0.010 ml.
19 ^{12}C-amino acids - ^{14}C-amino acid (each 8×10^{-4}M)	0.010 ml.
^{14}C-amino acid (neutralised)	0.010 ml.
tRNA in D'	0.070 ml.
Synthetases or S-150 in D'	0.020 ml.
H_2O	0.030 ml.
total nominal volume	0.225 ml.

nominal final conditions:

3.8M KCl, 1.4M NaCl, 0.04M KOAc, 0.03M Tris-HCl, pH 8.0,

1.3mM ATP, 8nmoles of each ^{12}C-amino acid,

1 - 20 nmoles ^{14}C-amino acid, 0.1 - 0.3 mg. tRNA,

0.2 - 0.4 mg. protein of S-150 or synthetases preparation.

111 Bulk preparation of aminoacyl - tRNA

^{14}C- or ^{35}S- aminoacyl - tRNA was prepared by incubation of one radioactively labelled amino acid with or without the other 19 ^{12}C-amino acids in a large scale incubation mixture increased proportionately from that described in table 4.

After incubation at 37°C the reaction was terminated at 0°C with the addition of 0.1 volume potassium acetate, pH 4.5, 20% $^{W}/_{V}$, and the mixture was dialysed for 1hr. against a large volume of 5mM potassium acetate, pH 4.5, and 5mM 2-mercaptoethanol. This was followed by a normal phenol extraction and precipitation of tRNA with 0.1 volume of potassium acetate, pH 4.5, 20% $^{W}/_{V}$, and 2 volumes of 95% ethanol.

The precipitated aa-tRNA was thoroughly freed of free amino acid by alternately dissolving in 1M potassium acetate, pH 4.5, and precipitating with ethanol. Alternatively, the aa-tRNA was passed through a short DEAE - cellulose column under acidic conditions and eluted with 1M NaCl.

If the aa-tRNA was required for further assays it was dissolved in 5mM potassium acetate, pH 4.5, containing 5mM 2-mercaptoethanol, dialysed thoroughly against the same buffer and frozen and stored at -20°C. If the aa-tRNA was required for chromatography it was dissolved in the starting buffer and applied immediately to the column.

iv Ribosomal binding :White & Bayley (1972a):

The reaction mixture is shown in table 5. The aa-tRNA was added last to the complete mixture which was maintained at 0°C.

After 30min. at 37°C the reaction was terminated by the addition of 4ml. of solution D' at 0°C. The Millipore filter, type HA, 0.45 micron, was washed with 4ml. of D' at 0°C and the incubation mixture filtered under gentle suction. The filter was then washed twice with 4ml. of D' at 0°C, dried and counted as previously described.

v Methionyl - puromycin formation

The procedure was based on that of Bretscher & Marcker (1966). The reaction mixture was identical to that shown in table 5 with the inclusion of puromycin to a final concentration of 1mM.

After 30min. at 37°C the reaction was terminated by the addition of 1ml. of 0.1M Na_2HPO_4, pH 8.1, containing 10mM methionine, and 1.5ml. of ethyl acetate (saturated with phosphate buffer). The two phases were thoroughly mixed on a vortex mixer and then were separated by centrifugation. The upper ethyl acetate phase was carefully removed and counted in a Beckman scintillation counter with Aquasol as the scintillation fluid.

Table 5

Reaction mixture for H.cutirubrum ribosomal binding *

Solid KCl	26 mg.
3.4M KCl, 0.12M MgAc, 0.15M Tris-HCl, pH 8.0	0.025 ml.
1M MgAc	0.006 ml.
Polyribonucleotide (mg./ml. H_2O)	0.010 ml.
aa-tRNA in 5mM NAc, pH 4.5	0.015 ml.
Ribosomes in D'	0.020 ml.
H_2O	0.049 ml.
total nominal volume	0.125 ml.

* reaction mixture with inclusion of puromycin (and GTP if necessary) suitable for not - puromycin formation

nominal final conditions :

3.5M KCl, 0.08M MgAc, 0.03M Tris-HCl, pH 8.0

polyribonucleotide 0.05mg., 5 - 50 pmoles aa-tRNA,

4 - 5 A_{260} units of ribosomes.

3 E.COLI

1 Aminoacyl-tRNA formation

The reaction mixture is shown in table 6 and is modified from that of Marshall et al.(1967).

After 20min. at 37°C the reaction tube was treated as for the H.cutirubrum system. For bulk aminoacylation of H.cutirubrum tRNA the procedure, after incubation, was followed exactly as described for the H.cutirubrum system.

11 Ribosomal binding (Nirenberg & Leder (1964).

The reaction mixture is shown in table 7. The aa-tRNA was added last to the complete mixture which was maintained at 0°C.

After 20min. at 24°C the reaction was terminated by the addition of 4ml. of 0.02M Tris-HCl, pH 7.2, 0.02M MgAc, and 0.05M KCl at 0°C. A Millipore filter, type HA, 0.45 micron, was washed with 4ml. of the above buffer and the incubation mixture was filtered under gentle suction. The filter was washed with 2 x 4ml. of the buffer at 0°C and dried and counted as previously described.

111 Methionyl - puromycin formation

The procedure was followed exactly according to Leder & Primrose (1966).

Table 6

Reaction mixture for E.coli aminoacylation

1M Tris-HCl, pH 7.5	0.005 ml.
0.5M $MgCl_2$	0.005 ml.
0.1M Na ATP	0.010 ml.
0.05M Na PEP	0.030 ml.
Pyruvate kinase (1mg./ml. H_2O)	0.010 ml.
19 ^{12}C-amino acids - ^{14}C-amino acid (each 8×10^{-4}M)	0.010 ml.
^{14}C-amino acid (neutralized)	0.010 ml.
tRNA in H_2O	0.100 ml.
S-150 in 0.03M KCl, 0.01M $MgCl_2$, 0.04M Tris-HCl, pH 7.8	0.020 ml.
H_2O	0.050 ml.
total volume	0.250 ml.

final conditions :

2mM KCl, 10mM $MgCl_2$, 20mM, Tris-HCl, pH 7.5

4mM ATP, 6mM PEP, 0.01mg. pyruvate kinase,

8 nmoles of each ^{12}C-amino acid, 1 - 20 nmoles ^{14}C-amino acid,

0.1 - 0.3 mg. tRNA, 0.5 mg. protein of S-150

Table 7

Reaction mixture for E.coli ribosomal binding

0.5M KCl, 0.2M MgAc, 0.2M Tris-HCl, pH 7.2	0.005 ml.
Polyribonucleotide (5mg./ml. H_2O)	0.005 ml.
aa-tRNA in 5mM KAc, pH 4.5	0.015 ml.
Ribosomes in BRS.	0.010 ml.
H_2O	0.015 ml.
total volume	0.050 ml.

BRS. = 0.03M KCl, 0.01M MgAc, 0.04M Tris-HCl, pH 7.5

final conditions:

0.05M KCl, 0.02M MgAc, 0.02M Tris-HCl, pH 7.2

0.025 mg. of polyribonucleotide

5 - 50 pmoles aa-tRNA

2 A_{260} units of ribosomes.

4 Separation techniques

A Column chromatography

i Sephadex gel filtration

G-75 and G-100 Sephadex columns were prepared in high salt, solution D', and low salt, 0.01M $MgCl_2$, Tris-HCl, pH 7.5, or 0.01M KAc, pH 4.5, in the same manner. 15g. of Sephadex was swollen in a large excess of buffer by boiling for 3hrs. and leaving at 4°C for at least 24hrs. Frequent settling and decanting was employed at 4°C in order to prevent salt crystallisation and to remove fines.

A 2.5 x 45 cm. glass column was packed and equilibrated with the appropriate buffer. The sample (ribosomes, S-60, S-150 tRNA or aa-tRNA) was applied in and eluted by the same buffer at 4°C with a flow rate of approximately 30 - 40 ml./hr.

ii DEAE - Sephadex (diethylaminoethyl-Sephadex)

DEAE - Sephadex, activated according to Nishimura et al. (1967), was packed into a 2.5 x 45 cm. glass column and equilibrated with 0.375M NaCl, 0.05M NaOAc, pH 4.5, 0.01M $MgCl_2$ and 0.01M 2-mercaptoethanol. Aminoacyl-tRNA was applied in the same buffer and was eluted with a linear salt gradient at 4°C with a flow rate of 30ml./hr.

iii BD - cellulose (benzoylated diethylaminoethyl - cellulose)

A glass column was packed with BD - cellulose according to Gillam et al. (1971). After thorough washing with 2M NaCl it was equilibrated with 0.45M NaCl, 0.01M $MgCl_2$, 0.05M NaOAc, pH 4.5, and 5mM 2-mercapto-ethanol. tRNA or aa-tRNA was applied in the same buffer and was eluted either by gradient or stepwise elution. Complete details of elution conditions are proved in the Results section.

iv Reversed phase chromatography (RPC - 5)

A 0.77 x 58 cm. RPC-5 column was prepared according to Pearson et al. (1971). The aa-tRNA was applied in 0.25M NaCl, 0.001M $MgCl_2$, 0.01M NaOAc, pH 4.5, and 5mM 2-mercaptoethanol at 7°C with a flow rate of 40ml./hr. Elution utilised a linear salt gradient, 0.25M - 0.75M NaCl, at the same temperature and flow rate.

v Assay and recovery of aa-tRNA

Column fractions were assayed either according to Griffiths (1970) or Nishimura et al. (1967). Aminoacyl - tRNA was precipitated with the addition of 0.1 volume of potassium acetate, pH 4.5, 20% $^{w}/_{v}$, and 2 volumes of 95% ethanol. The precipitate was collected by centrifugation or by retention on a Millipore filter. The latter filtration proved more efficient when dealing with small amounts of tRNA.

B High voltage electrophoresis

Resolution of methionine, puromycin and their derivatives was achieved by high voltage electrophoresis on Whatman 3MM paper in 0.5% pyridine - 5% acetic acid, pH 3.5, with an applied voltage of 50 V/cm. for 1.5hrs. Puromycin was detected under ultraviolet light and compounds with a free alpha amino group were detected with 5% ninhydrin - acetone. Radioactive compounds were detected by cutting the Whatman paper into 3 x 1 cm. strips and counting in the scintillation counter with omnifluor as the scintillation fluid.

Samples were eluted from the Whatman paper with ethyl acetate - methanol - water (1 : 1 : 1) and dried under vacuum at room temperature.

5 Chemical analyses

i Protein : Lowry et al. (1951)

To the sample in 1ml. of water was added 5ml. of a solution composed of 50ml. of 2% Na_2CO_3 in 0.1N NaOH and 1ml. of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% potassium tartrate. After 15min. 0.5ml. of 1N Folin reagent was added rapidly and shaken. The absorbance at 500nm. was then taken after a period of 30min. Bovine serum albumin was used as the standard (0 - 0.4mg. protein).

ii DNA : Burton (1968)

A slight modification was made in the composition of the diphenylamine reagent. It consisted of 3g. diphenylamine, 12ml. concentrated H_2SO_4, 100ml. glacial acetic acid and 0.1ml. of 1.6% acetaldehyde per 20ml. of diphenylamine reagent.

To the sample, previously hydrolysed at 70°C for 15min. in 0.5N perchloric acid, was added 2 volumes of diphenylamine reagent. The absorbance at 600nm. was then taken after a period of 16 - 20 hrs. at 30°C. Hydrolysed salmon sperm DNA was used as the standard (0 - 0.25mg. DNA).

iii RNA : Schneider (1957)

To the sample in 1.5ml. of water was added 1.5ml. of a solution composed of 1g. orcinol in 100ml. of concentrated HCl to which had been

added 0.5g. $FeCl_3$. The absorbance at 660nm. was then taken after a period of boiling for 20min. Adenosine or E.coli tRNA was used as the standard (0 - 0.12mg. RNA).

iv Total phosphorus ;Ames (1966)

The sample in 0.01ml. of water was firstly ashed after the addition of 0.03ml. of 10% magnesium nitrate in 95% alcohol and then completely hydrolysed by the addition of 0.3ml. of 0.5N HCl and boiling for 15min. To the cooled sample was added 0.7ml. of a solution containing 10% ascorbic acid - 0.42% ammonium molybdate in 1N H_2SO_4 (1 : 6). The absorbance at 820nm. was then taken after a period of 1hr. at 37°C. Analytically pure KH_2PO_4, similarly ashed and hydrolysed, was used as the standard (0 - 0.1mg. KH_2PO_4). The weight of RNA was taken as 11 x the weight of phosphorus.

v Ribosomes

The concentration of halophilic ribosomes was calculated from the absorbance at 258nm. using $E^{1\%}_{1cm.} = 158$ (Bayley & Kushner, 1969).

For both ribosomes and tRNA, 1 A_{260} unit was taken as the amount of material which in 1ml. would yield a value of 1.0 for the absorbance measured at 260nm. in a cuvette of path length 1cm.

III MATERIALS

Yeast extract and casamino acids were obtained from Difco Labs.; Inorganic salts, TCA, sodium dodecyl sulphate and L-amino acids from Fisher Scientific Ltd., and Canlab Ltd.; Bovine serum albumin, salmon sperm DNA, alumina (neutral 7) and trisodium 2-phoshoenol-pyruvate from Calbiochem Corp.; Tris from Sigma Chemicals Ltd.; 2-mercaptoethanol from Eastman Chemicals Ltd.; crystalline pancreatic RNAse and electrophoretically purified DNAse from Worthington Biochemical Corp.; pyruvate kinase (rabbit muscle), E.coli B tRNA (stripped), E.coli W cells (early log), disodium adenosine 5' - triphosphate and sodium guanosine 5' - triphosphate from P-L Biochemicals Inc.; antifoam A from Dow Corning Co.; puromycin dihydrochloride from Nutritional Biochemicals Ltd.; omnifluor from Beckman Ltd.; perchloric acid and Plaskon from Allied Chemicals Ltd.; adogen 464 from Aldrich Chemicals Ltd.; Sephadex gels from Pharmacia Ltd.; DE-cellulose from Schwarz Bioresearch Inc.; T_1-RNAse, poly (GU, 1:1) and ApUpG from Miles Laboratories; radioactivity labelled amino acids (NEN. sp. act. ^{35}S-methionine greater than 100 C/mmole) from New England Nuclear Ltd.

IV RESULTS

1 Initial chromatography of H.cutirubrum met-tRNA

Experiments to determine whether translational initiation is mediated through the formylatable species of H.cutirubrum $tRNA^{met}$ necessitated a clean separation of the two species. The procedure for achieving this was followed according to B.N.White (1970) and White & Bayley (1972c) and the resultant chromatogram is shown in figure 3. The result consistently differed from that previously reported in two ways; firstly, the A_{260} and radioactivity profiles were now concurrent and secondly there was a marked tailing of radioactivity throughout the 0.9M NaCl elution. Although the overall distribution of material vis. salt A_{260}, cpm. : ethanol A_{260}, cpm. agreed with the reported values, the same resolution was not being obtained.

The degree of resolution obtained in figure 3 was assessed by re-chromatography of the salt and ethanol peaks. The salt eluant, material taken from directly under the cpm. peak, showed good rechromatography in figure 4a where approximately 90% of the radioactivity reran with the 0.9M NaCl fraction. However, rechromatography of the ethanol eluant, as shown in figure 4b, resulted in a 1 : 1 distribution of cpm. between the salt and ethanol fractions. A comparison of the observed results with those reported previously, table 8, indicates the difference between the two chromatographic systems.

The tailing in figure 3 was considered to represent either an incomplete elution of the salt $tRNA^{met}$ or a mixture of the two species. In either case the implication was that a single ED-cellulose column would

Figure 3

Initial chromatography of ^{35}S-met-tRNA on BD-cellulose

1.5 x 26 cm. column, at 4°C with a flow rate of 20 ml./hr.

2ml. fractions, 5% TCA precipitation of selected fractions.

Applied : 66 A_{260} units, 3.3×10^5 cpm. (approx. 5 pmoles/A_{260}).

Stepwise elution : 0.45% NaCl equilibrating buffer, 0.9% NaCl, and 0.9% NaCl + 10% ethanol. All buffers contained 10mM $MgCl_2$, 50mM sodium acetate, pH 4.5, and 1mM 2-mercaptoethanol.

Recovery : complete A_{260} and 96% cpm.

Distribution of A_{260} : salt elution, 80% ; ethanol purge, 20%.

Distribution of cpm. : salt elution, 65% ; ethanol purge, 35%.

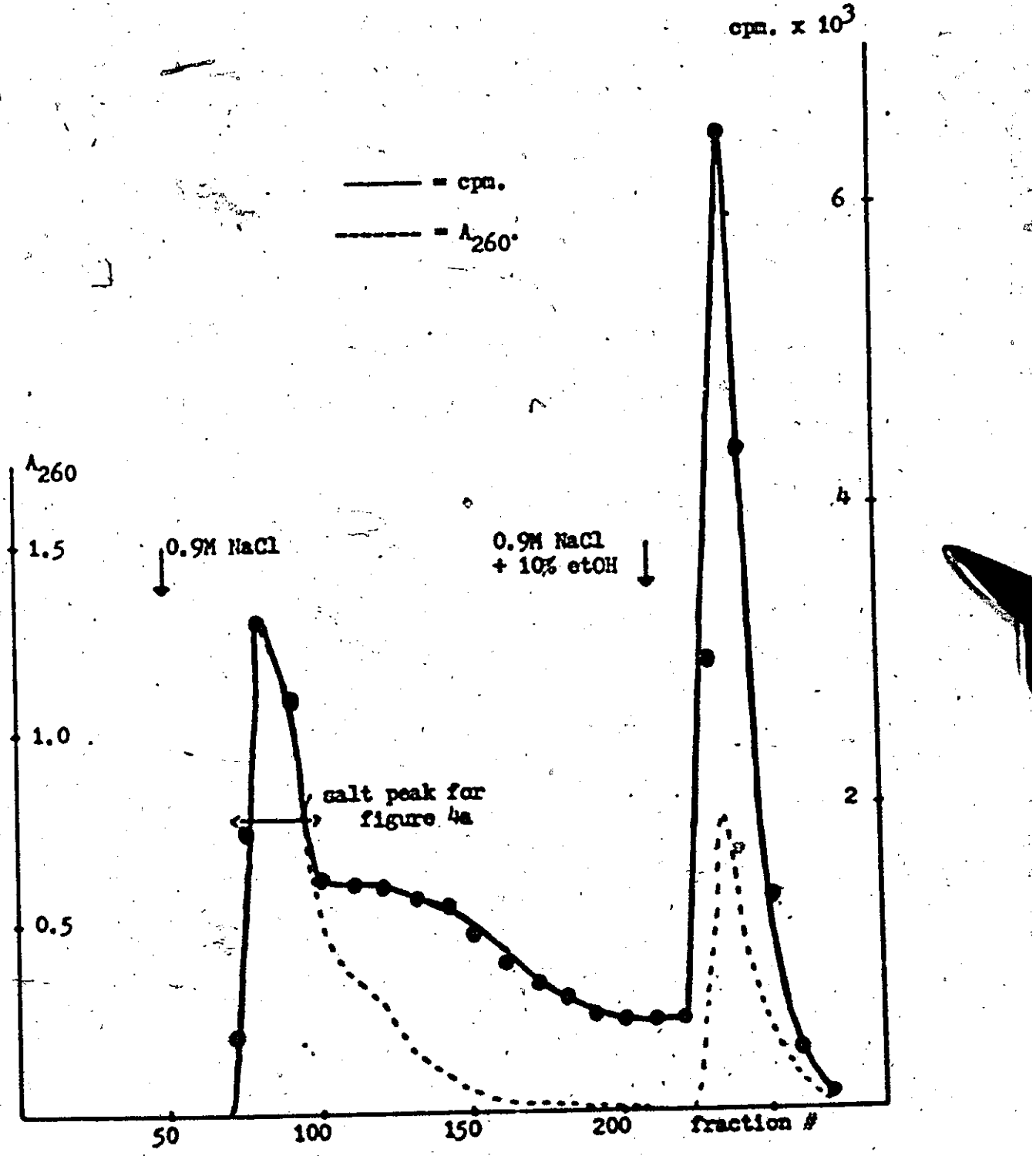

cpm. x 10^3
——— = cpm.
------ = A_{260}
A_{260}
1.5
1.0
0.5
0.9M NaCl
0.9M NaCl
+ 10% etOH
salt peak for
figure 4a
6
4
2
50
100
150
200
fraction #

Figure 4

Rechromatography of salt and ethanol peaks from figure 3

a Rechromatography of salt peak

Column dimensions and elution conditions as for figure 3.

Applied : approx. 2.1×10^4 cpm.

Distribution of cpm. : salt elution, 90% : ethanol purge, 10%.

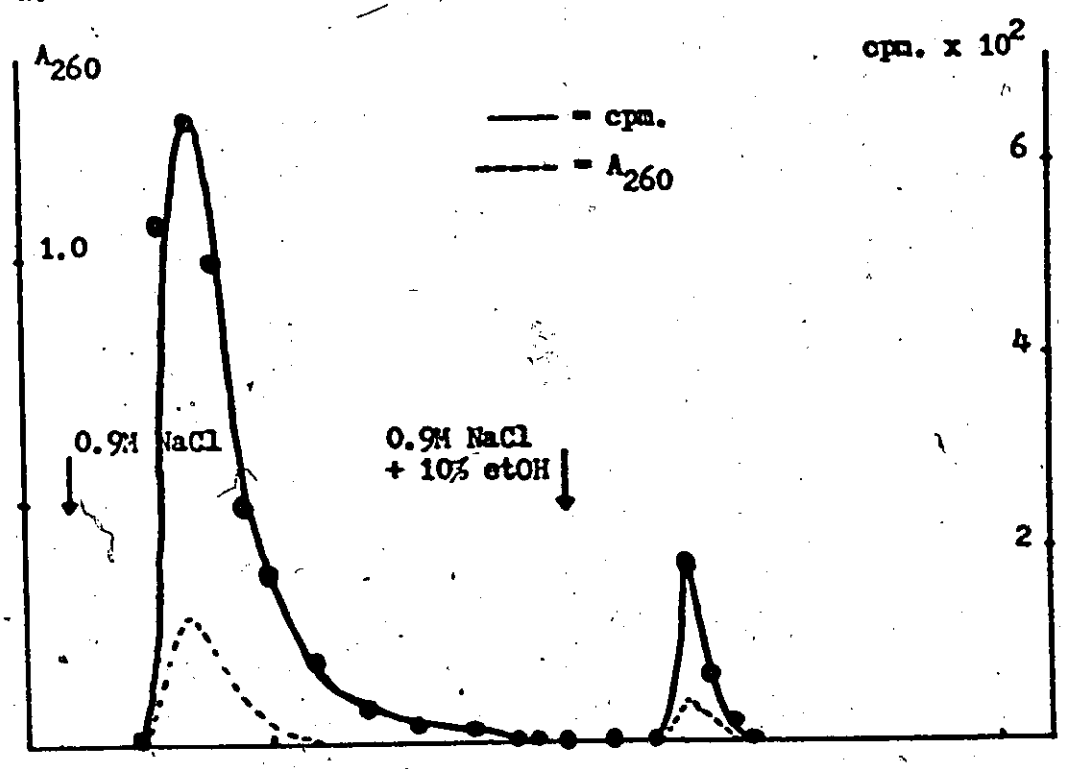
b Rechromatography of ethanol peak

Column dimensions and elution conditions as for figure 3.

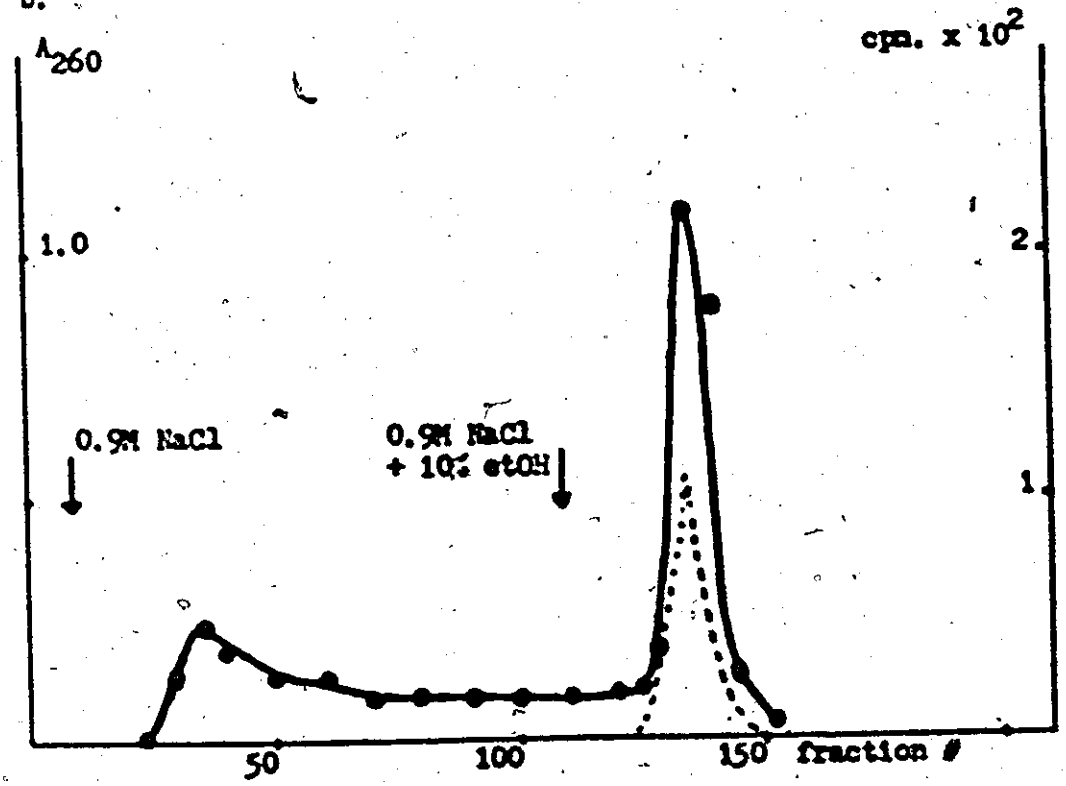
Applied : approx. 6.2×10^3 cpm.

Distribution of cpm. : salt elution, 50% : ethanol purge, 50%.

a.


A_{260}
cpm. x 10^2
—— = cpm.
- - - = A_{260}
1.0
6
4
2
0.9M NaCl
0.9M NaCl
+ 10% etOH

b.


A_{260}
cpm. x 10^2
1.0
2
1
0.9M NaCl
0.9M NaCl
+ 10% etOH
50
100
150
fraction #

not achieve resolution and that this would require at least one re-chromatography. This result in itself was not a major obstacle but in conjunction with the very low purity of the tRNA (prep. a, page 17) as exemplified in table 9, it made the chromatography appear a highly uneconomical procedure.

A hypothetical isolation procedure for the formylatable species of $tRNA^{met}$ at this stage would have been as follows:- 1g. wet weight of cells (42hr. growth) would yield approximately 2mg. of tRNA (purity of $tRNA^{met}$ approx. 0.3%) which would give approximately 12.5 nmoles of ethanol $tRNA^{met}$ (purity approx. 0.25%) after one rechromatography. This calculation would not include any loss of material on or between columns and assumed 100% separation of $tRNA^{met}$ species after one re-chromatography. It compared most unfavourably with results reported by RajBhandary & Ghosh (1969) where 1mg. of yeast tRNA yielded approximately 220 nmoles $tRNA^{met}_{fmet}$ (purity approx. 90%). The estimate of tRNA purity was based on the acylation of amino acid per A_{260} unit of tRNA, with 1800 nmoles indicative of 100% purity (Ghosh et al, 1971).

These initial results had failed to achieve an adequate resolution and were particularly disappointing as regards the ethanol species. Therefore it was felt that the <u>in vitro</u> experiments should be laid aside until such time as the $tRNA^{met}$ species became available in sufficient yield and purity. Two approaches were adopted to achieve this goal; firstly, BD-cellulose chromatography was studied in greater detail to obtain a suitable resolution and secondly, methods were attempted to obtain a purer preparation of tRNA.

Table 8

Comparison of observed chromatography (figures 3 & 4) with reported chromatography (B.N.White, 1970)

	% formylatable	% distribution on BD-cellulose			
		A_{260}		cpm.	
		Salt	EtOH	salt	etOH
REPORTED					
Unfractionated met-tRNA	50	86	14	67	33
Rechromatography of salt met-tRNA	4	83	17	99	1
Rechromatography of EtOH met-tRNA	80	27	73	25	75
OBSERVED					
Unfractionated met-tRNA		80	20	65	35
Rechromatography of salt met-tRNA		80	20	90	10
Rechromatography of EtOH met-tRNA		-	100	50	50

Table 9

Comparison of H.cutirubrum tRNA with tRNA from other organisms

Organism	Reference	Yield (mg.tRNA per g. of organism)	Acylation (pmole met per A_{160} unit)
E.coli	1	1.8mg.	45
"	2	no value	83
Yeast	3	1.0mg.	29
Wheat germ	4	0.9mg.	40
"	5	no value	27
Drosophila	6	1.0mg.	45
Guinea pig	7	no value	47
H.cutirubrum		2.0mg.	5

1 von Ehrenstein. (1968)
2 Nishimura et al. (1967)
3 Takeishi et al. (1968)
4 Dudock et al. (1969)
5 Ghosh et al. (1971)
6 B.N.White. (personal communication)
7 Caskey et al. (1967)

2. BD-cellulose chromatography and the preparation of tRNA

BD-cellulose (benzoylated Dnae-cellulose) was introduced by Gillam et al. in 1967 and has since proven of great value for the isolation of isoaccepting species of tRNA. In studies concerning initiation it has provided the separation of initiator and propagator $tRNA^{met}$ in a number of organisms, including, E.coli, yeast, mouse liver, mouse ascites tumour cells (Smith & Marcker, 1970) and wheat embryo (Leiss & Keller, 1970). It functions as an anion exchange column with an increased affinity for aromatic groups and useful variables have been temperature, pH, and concentration of Mg^{2+} ion (Gillam et al, 1971).

In light of the overall similarity between the observed and reported chromatography, as displayed in table 8 (page 41), it was felt that only slight modification was necessary for achieving a clean separation. However, variations in concentration of Mg^{2+} ion, temperature, pH and length of column did not effect a suitable resolution with stepwise elution of 0.9M NaCl or greater. This lack of resolution was not due to the tRNA preparation since the procedure was followed according to B.N.White (1970) and variations in this procedure e.g. cells harvested in different phases of the growth cycle, cells used directly or frozen at -40°C before use, tRNA vacuum dried or freeze dried (page 17) had no effect on the chromatography. Similarly, variations in the aminoacylation procedure prior to column application had no effect on chromatographic resolution. At this stage there was no apparent reason why the stepwise elution conditions of B.N.White (1970) were not affording a clean separation.

It was possible however that the poor resolution was a consequence of the BD-cellulose preparation currently in use and that with this par-

ticular batch, the $tRNA^{met}$ species eluted at different salt concentrations than those previously reported. Therefore, a salt gradient was used to determine the exact concentrations required for elution and this is shown in figure 5. Three radioactivity peaks were observed at 0.57M NaCl, 0.67M NaCl, and 0.9M NaCl respectively, the last peak being concentrated with an ethanol purge. This result compared favourably with a similar gradient elution described by B.N.White (1970), the only differences being that in the latter case only a slight shoulder preceded the 0.67M NaCl peak and the final peak was eluted with an ethanol purge without a prior 0.9M NaCl elution step. The profile in figure 5 suggested that in all the previous attempts, stepwise elution of 0.9M NaCl or greater would have caused the release of the third peak during the salt elution and this would have contributed to the marked tailing, cf. figure 3, page 37.

Stepwise elution using 0.77M NaCl was selected as a result of figure 5 and this provided a clean separation of the $tRNA^{met}$ species as illustrated in figure 6a. The tailing was negligible under these conditions and rechromatography of the ethanol species was approximately 97%. It appeared that the earlier lack of resolution was due to a weaker binding of the ethanol species than that observed by B.N.White (1970) and that this was a consequence of the different BD-cellulose preparations, probably as a difference in the degree of benzoylation. The use of 0.77M NaCl stepwise elution with this BD-cellulose preparation provided a routine separation of the $tRNA^{met}$ species.

The importance of standardising a BD-cellulose preparation before its application in the separation of tRNA species was evident from the preceding chromatography. However a clearer illustration of this was

Figure 5

Gradient elution of ^{35}S-met-tRNA on BD-cellulose

1.5 x 26 cm. column, at 4°C with a flow rate of 20 ml./hr.

2ml. fractions, 5% TCA precipitation of selected fractions.

Applied : 115 A_{260} units, 1.8 x 10^5 cpm. (approx. 10 pmoles/A_{260}).

Gradient elution as shown opposite with buffers containing 10mM $MgCl_2$, 50mM NaOAc, pH 4.5, and 1mM 2-mercaptoethanol.

Recovery : complete A_{260} and 85% cpm.

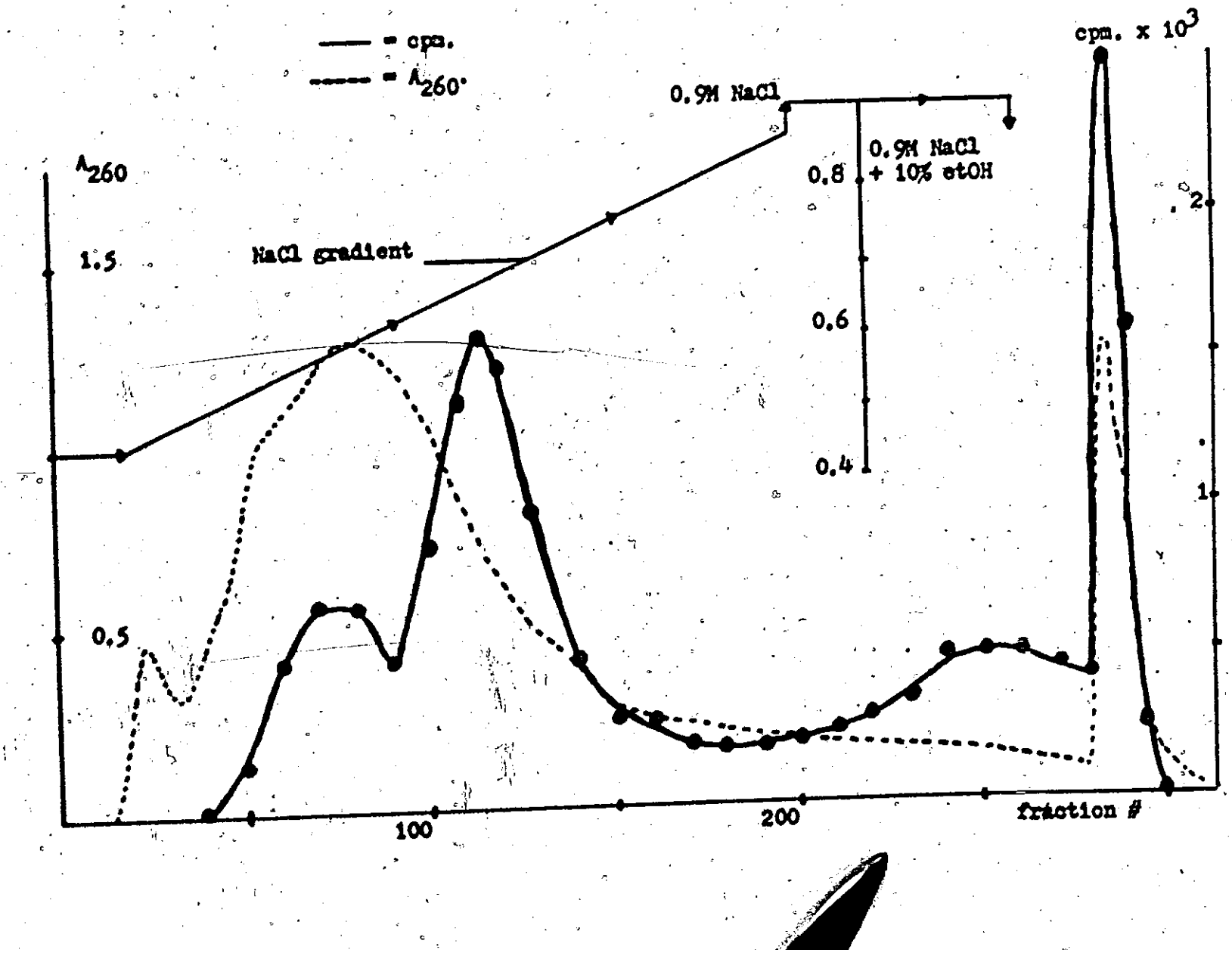
Distribution of A_{260} : salt elution, 92% ; ethanol purge, 8%

Distribution of cpm. : 0.57M NaCl peak, 14%

0.67M NaCl peak, 50%

0.9M NaCl peak, 21%

0.9M NaCl + 10% ethanol peak, 15%


—— = cpm.
----- = A_{260}
0.9M NaCl
A_{260}
1.5
NaCl gradient
0.5
0.9M NaCl
+ 10% etOH
0.8
0.6
0.4
cpm. x 10^3
2
1
100
200
fraction #

provided by a later batch of BD-cellulose. Figure 6b involved the use of a different commercial preparation than those previously used and the improvement in separation over that in figure 6a is most noticeable in the enhanced separation of absorbance and radioactivity profiles. The use of 0.9M NaCl stepwise elution with this batch of BD-cellulose did not differ significantly from figure 6b and therefore it is possible to directly compare the initial chromatography of figure 3 with that of figure 6b and to ascribe the difference to the particular BD-cellulose preparation in use.

In conjunction with the BD-cellulose chromatography, efforts were being made to improve the purity of the transfer RNA preparation. The tRNA used thus far was that described by Griffiths & Bayley (1969) and listed as preparation a. (page 17). It involved a long multi-step procedure and resulted in tRNA of low yield and purity which contained approx. 50% nucleic acid with no acceptance activity and a low M.W. inhibitor of protein synthesis (B.N.White, 1970). Two approaches were adopted to obtain a better preparation. The first entailed an investigation into the causes of the poor yield, the nature of the impurities and attempts at their removal whilst the second involved different preparative procedures for obtaining the initial tRNA product.

A G-100 Sephadex analysis of the preparative procedure, figure 30 shown in the appendix, served to indicate that considerable losses of material occurred as a result of the centrifugation step to remove the ribosomes (page 14) and during the pH 5 precipitation of synthetases (page 15). This material was likely to be tRNA since Elson (1964) showed that 1 - 3% of the RNA of purified E.coli ribosomes was tRNA and table 10 showed that the tRNA for six randomly selected amino acids was present in

Figure 6

Stepwise elution of ^{35}S-met-tRNA on BD-cellulose using a lower NaCl concentration

a 0.77M NaCl stepwise elution

1.5 x 26 cm. column, at 4°C with a flow rate of 20 ml./hr.

2ml. fractions, 5% TCA precipitation of selected fractions.

Applied : 86 A_{260} units, 1 x 10^5 cpm. (approx. 8 pmoles/A_{260}).

Stepwise elution as shown opposite with all buffers containing 10mM $MgCl_2$, 50mM NaOAc, pH 4.5, and 5mM 2-mercaptoethanol.

Recovery : complete A_{260} and cpm.

Distribution of A_{260} : salt elution, 85% ; ethanol purge, 15%

Distribution of cpm. : salt elution, 60% ; ethanol purge, 40%

b 0.77M NaCl stepwise elution with a different BD-cellulose prep^n.

Column dimensions and elution conditions as for figure 6a.

Applied : 42 A_{260} units, 6.4 x 10^4 cpm. (approx. 5.5 pmoles/A_{260}).

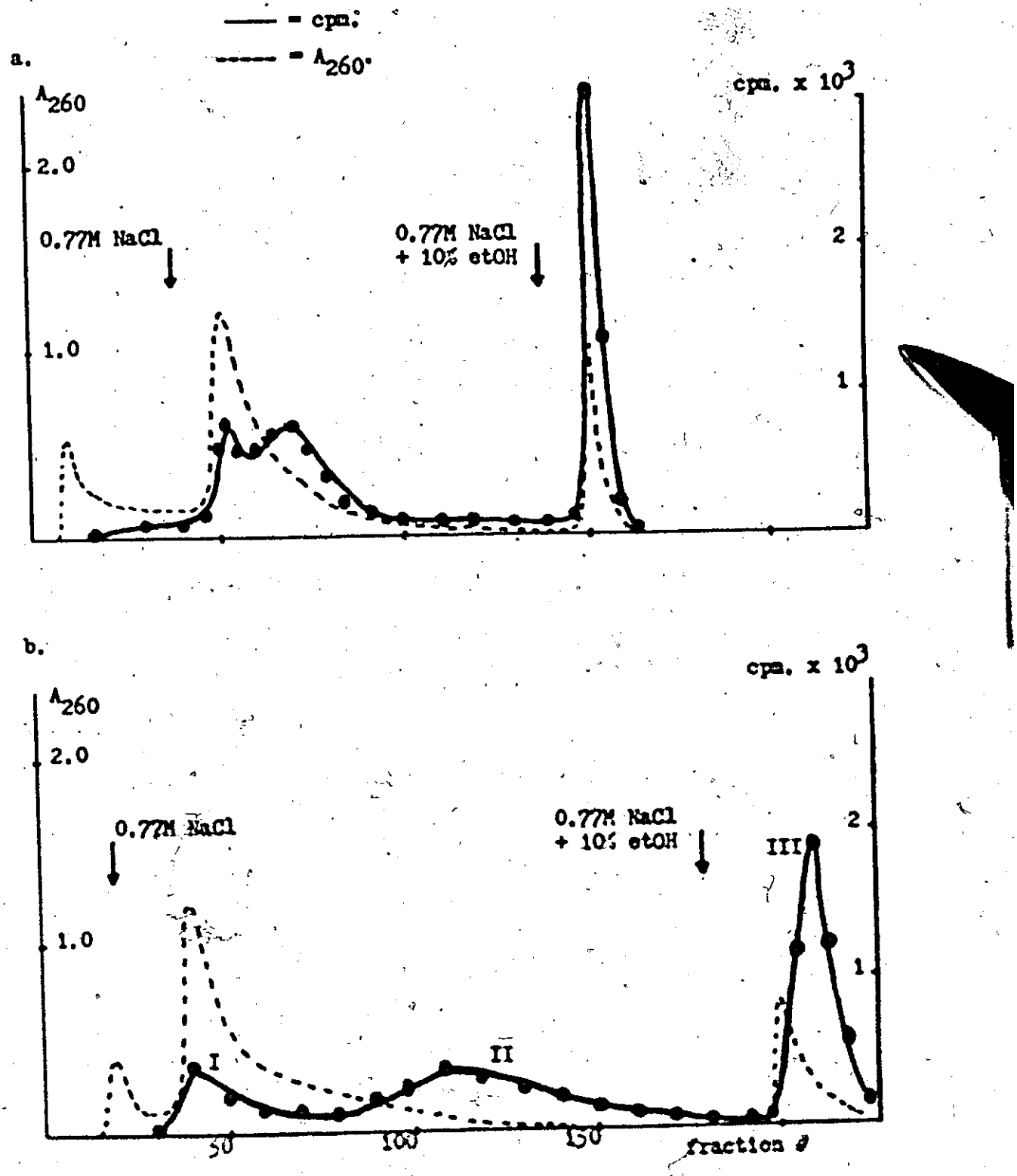
Recovery : complete A_{260} and cpm.

Distribution of A_{260} : salt elution, 75% ; ethanol purge, 25%

Distribution of cpm. : Peak I , 15%

Peak II , 35%

Peak III , 50%


—— = cpm.
---- = A260.
a.
A260
2.0
1.0
0.77M NaCl
0.77M NaCl
+ 10% etOH
cpm. x 10^3
2
1
b.
A260
2.0
1.0
0.77M NaCl
0.77M NaCl
+ 10% etOH
cpm. x 10^3
2
1
I
II
III
50
100
150
fraction #

a preparation of pre-incubated H.cutirubrum ribosomes. Similarly, aminoacylation assays, not shown, indicated that tRNA was present in the pH 5 synthetase preparation. This brief procedural investigation revealed substantial losses of tRNA and it did not include any loss prior to the S-60 stage (page 14) nor during the phenol extraction and subsequent isolation of tRNA.

G-100 Sephadex chromatography also provided a clear illustration of the impurities inherent in the tRNA and this is shown in figure 7. The profiles showed the crude tRNA to possess three major components, peak 1, of high M.W. eluting with the void volume, peak 2 in which resided only acceptor activity and peak 3 which was of low M.W. and reportedly contained an inhibitor of protein synthesis (B.N.White, 1970). The profile for H.cutirubrum tRNA is in sharp contrast to that for a commercial preparation of E.coli tRNA which showed approx. 95% of the A_{260} units as peak 2, figure 7c.

A chemical analysis of H.cutirubrum tRNA together with its G-100 Sephadex components is shown in table 11. The main impurities appeared to be protein, approx. 6%, localised in peak 1, DNA, approx. 20%, in peak 3 and RNA possessing no acceptor activity, approx. 65% in all three peaks. Peak 1 would possibly contain rRNA and protein whilst peak 2 would contain tRNA and a high proportion of degraded RNA. Peak 3 would contain a mixture of deoxyribo- and ribo-oligonucleotides of chain length greater than forty since they were not removed by thorough dialysis.

Attempts at chromatographic purification of the tRNA utilised Dease-cellulose, BD-cellulose and G-100 Sephadex but this only improved the purity by a factor of two. These procedures were most unsatisfactory

Table 10

Presence of tRNA in H.cutirubrum ribosomal preparation

amino acid	amount used per assay (nmoles)	specific activity (cpm/pmole)	NET pmole aa. per mg. of ribosomes.
^{14}C-met	1.95	540	14.4
^{14}C-phe	1.0	1000	1.5
^{14}C-val	1.98	530	3.0
^{14}C-leu	1.61	655	9.0
^{14}C-arg	1.79	590	43.5
^{14}C-isoleu	1.6	660	5.1

Normal H.cutirubrum charging system. (Table 4, page 22).

Pre-incubated ribosomes (page 15) were sedimented at 150,000g. for 2.5hr. resuspended in solution D' and used as the source of tRNA.

0.39mg. of ribosomal preparation per assay.

Peak 1, from a G-75 Sephadex column of a 100% $(NH_4)_2SO_4$ precipitate of an S-150 (page 15) was used as a source of synthetases (0.2 A_{260}/assay).

Each assay contained puromycin (0.025mg.).

Figure 7

Old tRNA (prep^{n}. a. page 17) on G-100 Sephadex

All columns, 2.5 x 45 cm., at 45°C with a flow rate of 36 ml./hr. 2ml. fractions were collected and columns were eluted with 10mM NaAc, Tris-HCl, pH 7.6

a Unfractionated H.cutirubrum tRNA (prep^{n}. a. (A), page 17)

Applied : 380 A_{260} units (40mg.).

Distribution : Peak 1 - 10%, Peak 2 - 50%, Peak 3 - 40%.

b Unfractionated H.cutirubrum tRNA (prep^{n}. a. (B), page 17)

Applied : 270 A_{260} units (20mg.).

Distribution : Peak 1 - 25%, Peak 2 - 50%, Peak 3 - 25%.

c E.coli B tRNA

Applied : 47 A_{260} units (2.5mg.).

Distribution : Peak 1 - 6%, Peak 2 - 94%.

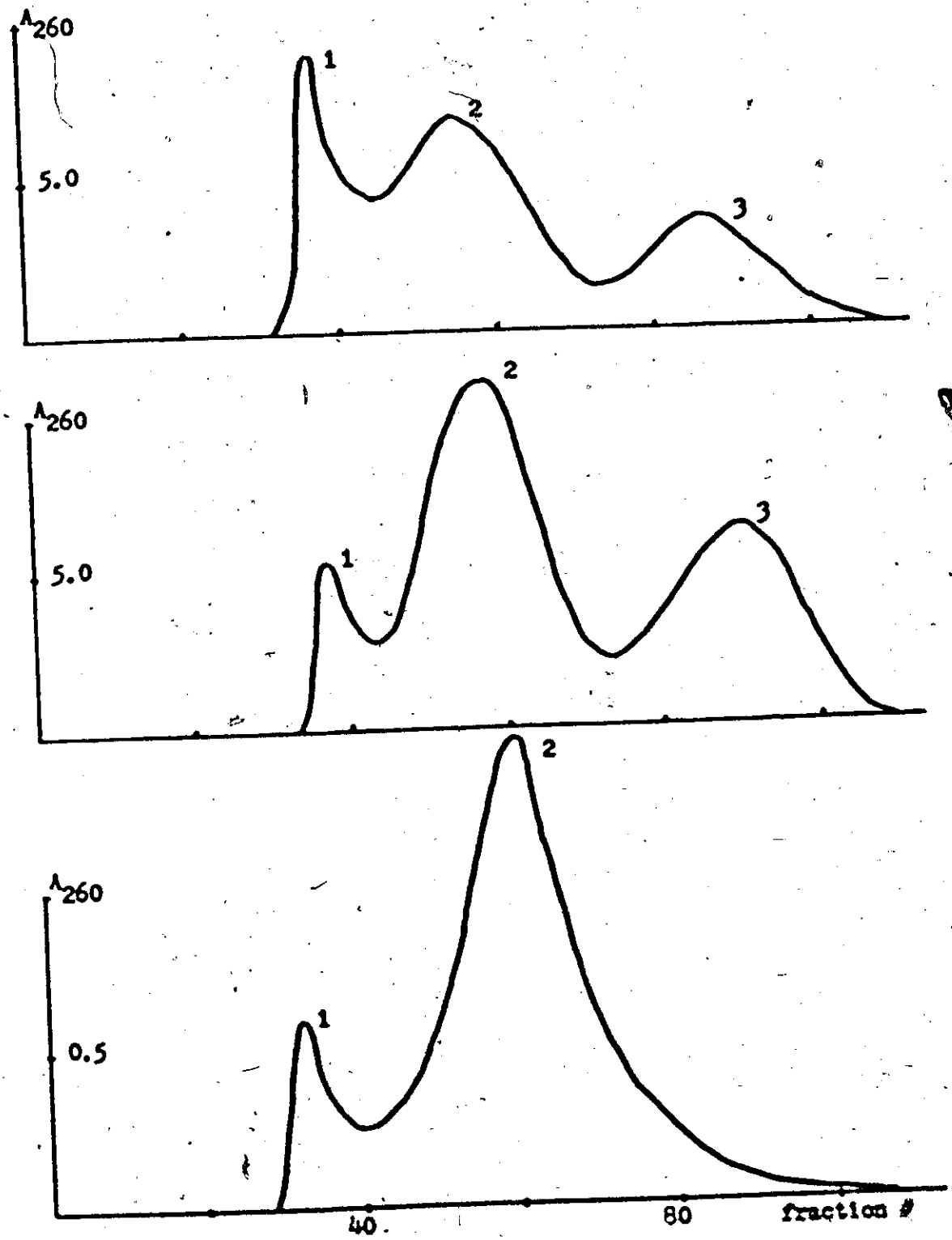

A260
5.0
1
2
3
A260
5.0
1
2
3
A260
0.5
1
2
40
80
fraction #

Table 11

Chemical composition of old tRNA (prep^{n}. a, page 17)

nucleic acid	% distribution on G-100 Sephadex	% protein	% DNA	% RNA	% increase in A_{260} upon hydrolysis
Crude tRNA		6	20	75	9
G-100 peak 1	20	30	-	70	12
G-100 peak 2	45	-	+	75	15
G-100 peak 3	35	-	50	60	3.5
E.coli tRNA		1.5	-	97	13

protein : method of Lowry (see page 32).

DNA : method of Burton (see page 32).

RNA : method of Schneider (see page 32).

hydrolysis : 19 hr. at 37°C in 0.5N NaOH.

and they indicated that, coupled with the losses which occurred during the preparative procedure, the best tRNA preparation available using these techniques would have been approximately 1mg. of tRNA (purity, approx. 17%) from 1g. wet weight of cells. It was therefore felt that emphasis had to be directed towards the preparation of an initially purer tRNA.

The best approach appeared to require a more direct phenol extraction with a subsequent chromatographic step for the removal of high M.W. nucleic acid. After a number of attempts along these lines a method was selected, preparation b. (page 18), which provided a reasonably pure tRNA and which suffered from only two drawbacks. Firstly, large volumes of buffer were required to dilute the salt concentration sufficient to allow a phenol extraction and secondly, the usual DEAE-cellulose chromatography was not possible due to the high viscosity of the total nucleic acid precipitate (30% DNA). An isopropanol fractionation (von Ehrenstein, 1968) proved adequate for the removal of high M.W. material and the contribution of J.Patel (B.Sc.) must be lauded for its insertion into the preparative procedure. A G-100 Sephadex analysis of this preparative procedure is provided in the appendix, (figure 31).

The new procedure resulted in a slight increase in yield, 1g. wet weight of cells yielding 2.2mg. of tRNA, and of this material 92% eluted as peak 2 on G-100 Sephadex; a percentage which compared most favourably with the E.coli tRNA in figure 7c. The purity was assessed according to the acceptance of amino acid and is shown in table 12. The new tRNA was approximately eight times purer than the old crude preparation; taking 130 pmoles of amino acid per A_{260} unit of tRNA for the old prepa-

ration (B.N.White, 1970) then the new preparation provided approx. 1100 pmoles. This value corresponded to approximately 60% pure tRNA and this was likely to be a minimum value since no attempt was made to optimize the acylation conditions for each amino acid.

The absence of DNA contamination and peak 3 material indicated that the tRNA would not be inhibitory in an in vitro polypeptide synthesising system and this assumption was shown to be correct by the results in table 13. In all respects the new tRNA was far superior to that previously used and it was therefore used in subsequent work. Its immediate application was the chromatography of ^{35}S-met-tRNA on BD-cellulose but, taking advantage of the high purity, the chromatographic behaviour of other aminoacyl-tRNAs was also analysed and these are shown in the appendix.

Table 12

Acceptance of amino acids, new tRNA v old tRNA

amino acid	amount used per assay (nmole)	NET pmole aa. accepted per A_{260} unit of tRNA.	
		OLD tRNA	NEW tRNA
methionine	10	4.9	27
arginine	1.8	12.5	110
glycine	4.0	10.3	115
isoleucine	1.6	9.6	60
leucine	1.6	9.4	64
phenylalanine	1.0	5.6	30
valine	2.0	10.8	83
tryptophan	4.0	3.8	51
asparagine	3.0	0.6	3.3

Normal H.cutirubrum charging system (Table 4, page 22).

Old tRNA values taken from B.N.White (1970).

New tRNA, 2 A_{260} units per assay.

0.2 A_{260} units synthetase preparation per assay. (peak 1 from G-75 Sephadex).

Table 13

Inhibition of polypeptide synthesis by H.cutirubrum tRNA

addition, A_{260} units of tRNA per assay.	pmoles of ^{14}C-phe in hot 5% TCA ppt.
- - - -	10.4
Old unfractionated tRNA 0.38 A_{260}	6.4
Old tRNA, G-100 Sephadex peak 3, 0.35 A_{260}	4.5
New unfractionated tRNA 0.35 A_{260}	15.3

Normal H.cutirubrum polypeptide synthesising system (Table 3, page 21).

S-150 used as source of synthetases and transferases and as a source of endogenous tRNA. (0.3mg. of protein in S-150 per assay).

1.4 A_{260} units of ribosomes per assay.

0.05mg. of poly U per assay.

0.55nmoles of ^{14}C-phe per assay (466 mC/mmole).

The control without added poly U has not been subtracted.

3 Properties of H.cutirubrum $tRNA^{met}$ species

1 Chromatographic resolution with new tRNA preparation

The new tRNA preparation provided sufficient yield and purity that it was now possible to take a more qualitative approach towards chromatography. In this respect it was relevant to assess the structural features of the $tRNA^{met}$ species which controlled their chromatographic behaviour.

The behaviour of the new tRNA preparation (preparation b, page 18) on BD-cellulose is illustrated in figure 8. A clean separation was evident and the ethanol met-tRNA rechromatographed as a single peak, figure 8b, to an extent of approximately 97% regardless of whether rechromatography utilised 0.7% or 0.9% NaCl eluants or an ethanol gradient. This excellent rechromatography, however, emphasised a difference in the proportion of met-tRNA species obtained with the new tRNA preparation. Previously, figures 3, 5 and B.N.White (1970), the salt eluted species had been greater than the ethanol species but now this was reversed and the distribution in figure 8a was peak I 2%, peak II 33% and peak III 65%.

This lower proportion of salt to ethanol cpm. might have resulted from incomplete acylation since in figure 8a less methionine was used for charging. Therefore the effect of varying the concentration of methionine on the extent of acylation and proportion of species was investigated and is shown in table 14. The effect was opposite to that expected since there appeared a preferential acylation of the salt species (peak II) when methionine was limiting but as the concentration increased so the ratio shifted in favour of the ethanol species (peak III). Control experiments involving the presence and absence of the 19^{12}C-amino

Figure 8

Rom tRNA preparation - ^{35}S-met-tRNA on BD-cellulose

1.5 x 26 cm. column at 4°C.

met-tRNA was applied in the 0.45M NaCl equilibrating buffer and then eluted by stepwise elution as indicated. All buffers contained 50mM sodium acetate, pH 4.5, 10mM $MgCl_2$ and 5mM 2-mercaptoethanol.

a. Unfractionated ^{35}S-met-tRNA

2ml. fractions collected at flow rate of 36 ml./hr.

Applied : 100 A_{260} units, 2.1 x 10^5 cpm. (approx. 17 pmoles/A_{260}).

6.8 A_{260} units of tRNA and 4 moles of methionine per normal assay were used for aminoacylation.

Recovery : complete A_{260} and cpm.

Distribution of cpm. : peak I - 2%, peak II - 33%, peak III - 65%.

b. Rechromatography of peak III from figure 8a

1.5ml. fractions collected at flow rate of 30 ml./hr.

Applied : 4 A_{260} units, 6.8 x 10^4 cpm. (approx. 100 pmoles/A_{260}).

Recovery : complete A_{260} and cpm.

Distribution of cpm. : peak I - 3%, peak II - 0%, peak III - 97%.

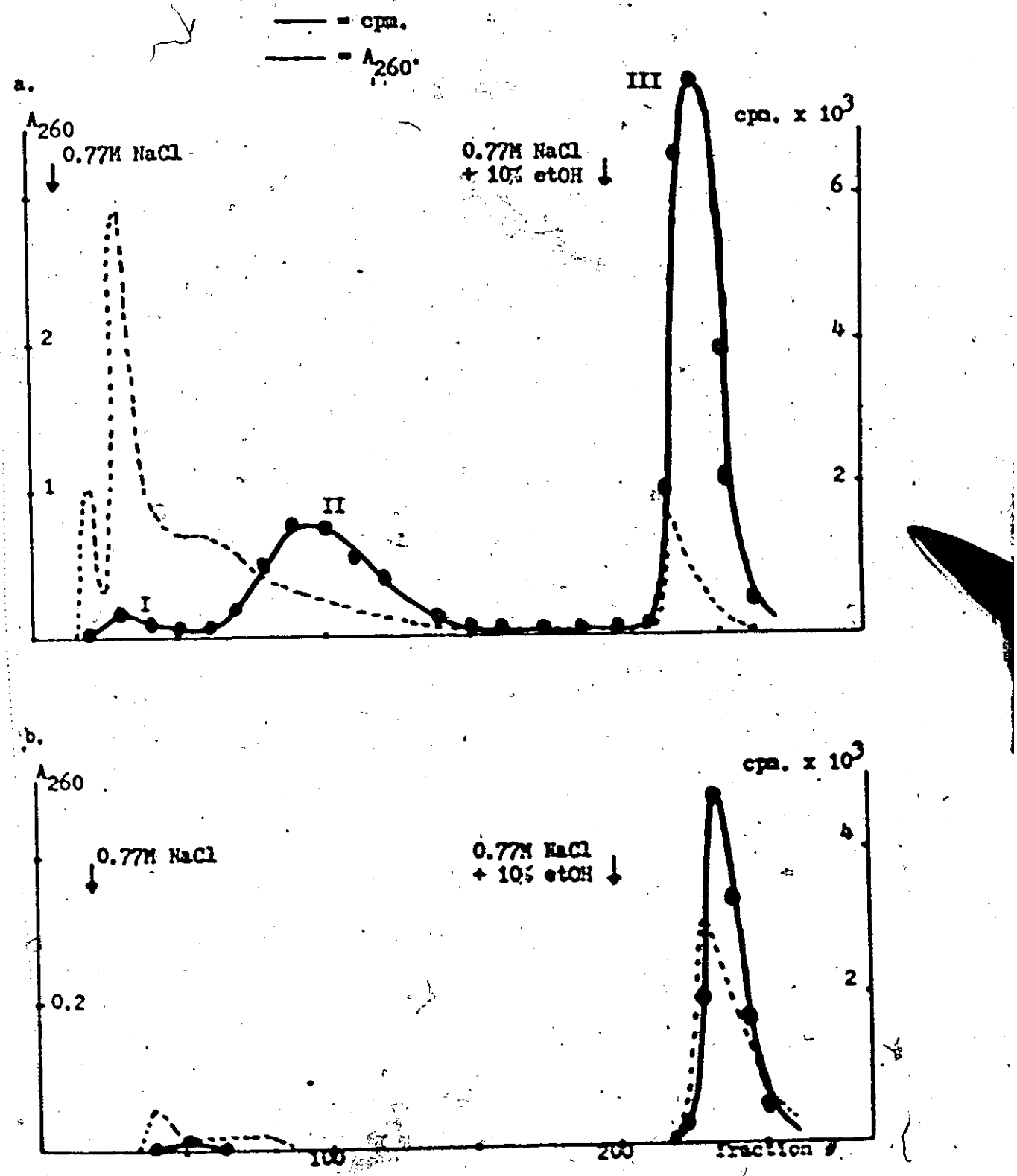

—— = cpm.
---- = A260.
a.
A260
0.77M NaCl
0.77M NaCl
+ 10% etOH
cpm. x 10^3
I
II
III
1
2
2
4
6
b.
A260
0.77M NaCl
0.77M NaCl
+ 10% etOH
cpm. x 10^3
0.2
2
4
100
200
fraction #

Table 14

The effect of methionine concentration on the proportion of $tRNA^{met}$ species observed on BD-cellulose

Aminoacylation *		BD-cellulose chromatography *		
nmole of met. available per normal assay	pmole of met. accepted per A_{260} of tRNA	% distribution of species peak I	peak II	peak III
0.004	0.016	2	70	28
2.0	13.0	2	37	61
4.0	19.5	2	33	65
6.0	23.7	no chromatography		
8.0	25.4	2	30	68

* separate experiments.

Normal H.cutirubrum charging system as described in table 4 (page 22).

6 - 7 A_{260} units of tRNA used per assay.

S-150 used as source of synthetase.

acids, different batches of tRNA and tRNA harvested from cells in early log phase together with the absence of any observed aggregate formation all supported the new proportions of met-tRNA species.

A possible explanation for the new ratio was that it reflected a basic difference between the old and new tRNA preparative procedures. Whereas the old tRNA possessed approx. 15% peak I material, figure 5 (page 46), the new preparation contained only 2 - 3%. This value seemed rather low for a distinct species and it could have represented an altered form of the ethanol species resulting from damage during extraction; in which case the old procedure was likely to contain more of this material. The exact nature of peak I, however, and the reasons why the two preparative procedures resulted in different proportions of species were not elucidated and remain undetermined.

The contribution of electrostatic and hydrophobic interactions towards met-tRNA species separation was investigated by comparison of BD-cellulose, Deae-Sephadex and reversed phase chromatography. Deae-Sephadex, illustrated in figure 9a, provided a slight purification in that the met-tRNA species eluted slightly ahead of the bulk of the tRNA, in a similar fashion to E.coli $tRNA^{met}$ chromatographed on Deae-Sephadex (Nishimura et al, 1967 ; Barker, 1970). This indicated that electrostatic interactions played only a minor role in the separation achieved on BD-cellulose, figure 8a, and that the latter was probably a consequence of hydrophobic attractions. The R.P.C.-5 column, shown in figure 9b, gave a very clean separation of species whose proportions were identical to those present on BD-cellulose. This was shown by the rechromatography of peaks II and III from the R.P.C.-5 column on BD-cellulose figures 10a and b, respectively. The order of elution on R.P.C.-5 was therefore

Figure 9

Deae-Sephadex and RPC-5 chromatography

a. ^{35}S-met-tRNA on Deae-Sephadex

2.5 x 30 cm. column, at 4°C with a flow rate of 30 ml./hr.

Applied : 4.3×10^3 A_{260}, 3×10^6 cpm. (approx. 0.1 pmole/A_{260}).

Two incubation mixtures were used for aminoacylation, a bulk incubation using ^{12}C-methionine and a small incubation using very high specific activity ^{35}S-methionine (2.9×10^3 mC/mmole).

NaCl gradient elution as shown opposite with buffers containing 50mM sodium acetate, pH 4.5, 10mM $MgCl_2$ and 10mM 2-mercaptoethanol.

7ml. fractions.

Recovery : approximately complete A_{260} and cpm., with no separation of met-tRNA species.

b. ^{35}S-met-tRNA on RPC-5

0.77 x 58 cm. column, at 7°C with a flow rate of 40 ml./hr.

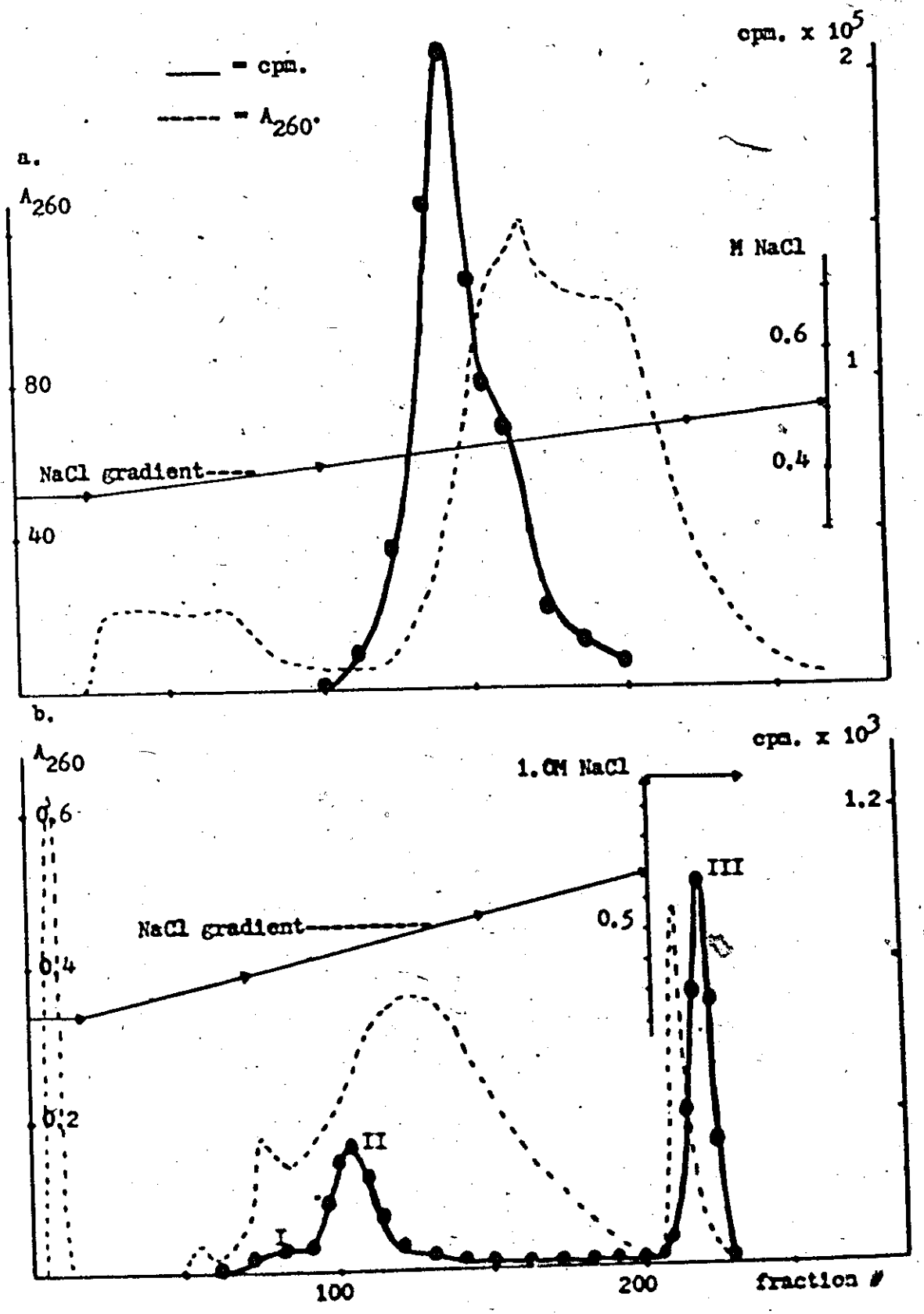
Applied : 46 A_{260} units, 1.9×10^4 cpm. (approx. 15 pmoles/A_{260}).

Aminoacylation using 7 A_{260} units of tRNA and 2 nmoles of methionine per normal assay.

NaCl gradient elution as shown opposite with buffers containing 10mM sodium acetate, pH 4.5, 1mM $MgCl_2$ and 5mM 2-mercaptoethanol.

Recovery : approx. 80% A_{260} and 85% cpm.

Distribution of cpm. : peak I - 2%, peak II - 41%, peak III - 57%.


cpm. x 10^5
2
1
0.6
0.4
M NaCl
A_{260}
80
40
NaCl gradient
a.
b.
cpm. x 10^3
1.2
0.5
1.0M NaCl
A_{260}
0,6
0,4
0,2
NaCl gradient
I
II
III
100
200
fraction #

the same as on BD-cellulose and it appeared to be governed by hydrophobic attractions.

It was clear from the previous chromatography that a difference in hydrophobic nature effected resolution but it was uncertain whether this was strictly dependent upon acylation. No detailed work was undertaken but one result did shed some light on this aspect. Deacylated tRNA was eluted from BD-cellulose under conditions suitable for separation of the charged species. The total salt tRNA was subsequently acylated and rechromatographed. The resultant distribution of salt: ethanol cpm. was 94 : 6 compared to 72 : 28 expected at this methionine concentration for unfractionated tRNA. The ethanol tRNA from the same deacylated tRNA chromatogram was similarly treated and in this case the distribution was 4 : 96 compared to the expected 40 : 60. The detailed results are shown in table 15. These results indicated that separation was not strictly dependent upon acylation but was a consequence of structural differences between the two $tRNA^{met}$ species which were probably increased by the attachment of amino acid.

The behaviour of the $tRNA^{met}$ species towards the different chromatographic systems, particularly the weak electrostatic and strong hydrophobic interactions, indicated their usefulness for the isolation of pure $tRNA^{met}$. Furthermore the consistency on BD-cellulose enabled the two separated species to be used for <u>in vitro</u> studies.

Figure 10

DD-cellulose chromatography of peaks II & III from RPC-5

a. Peak II from figure 9b rerun on DD-cellulose

1.5 x 27 cm. column, at 4°C with a flow rate of 36 ml./hr.

Applied : fraction #s 96-120 inc., approx. 2 x 10^3 cpm.

Stepwise elution as shown with all buffers containing 10mM $MgCl_2$, 50mM sodium acetate, pH 4.5, and 5mM 2-mercaptoethanol.

2ml. fractions.

Recovery : 90%.

Distribution : peak I - 3%, peak II- 95%, peak III - 2%.

b. Peak III from figure 9b rerun on DD-cellulose

1.5 x 26 cm. column, at 4°C with a flow rate of 39 ml./hr.

Applied : fraction #s 215-227 inc., approx. 2 x 10^3 cpm.

Stepwise elution as shown with all buffers containing 10mM $MgCl_2$, 50mM sodium acetate, pH 4.5, and 5mM 2-mercaptoethanol.

3ml. fractions.

Recovery : 100%.

Distribution : peak I - 10%, peak II - 0%, peak III - 90%.

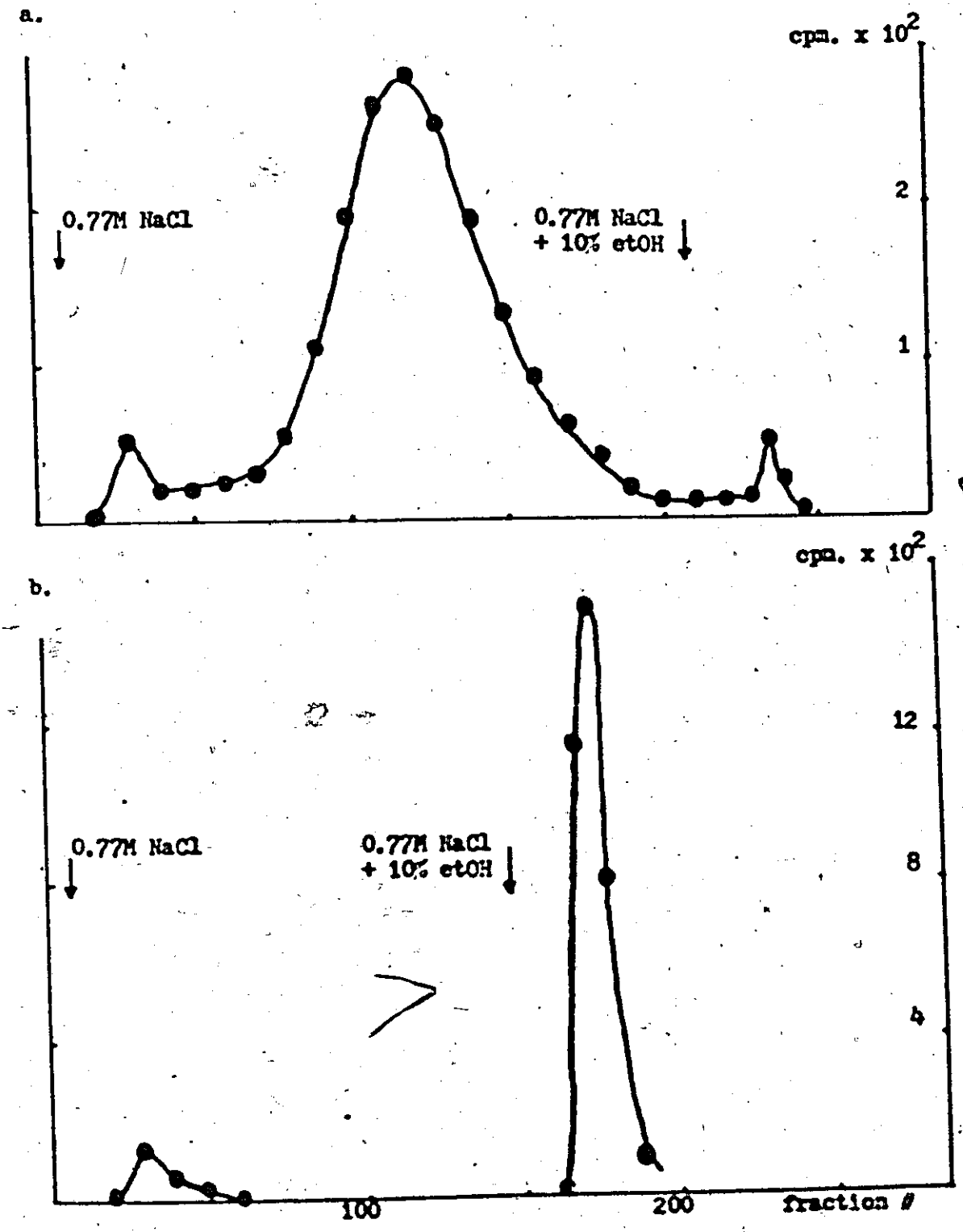

a.
cpm. x 10^2
2
1
0.77M NaCl
0.77M NaCl
+ 10% etOH
b.
cpm. x 10^2
12
8
4
0.77M NaCl
0.77M NaCl
+ 10% etOH
100
200
fraction #

Table 15

Behaviour of $tRNA^{met}$ on BD-cellulose

tRNA	distribution on BD-cellulose	aminoacylation * (pmole met./A_{260})
unfractionated		23
0.45M NaCl	14%	—
0.77M NaCl	78%	16
0.77M NaCl + 10% etOH	8%	100

The tRNA was subsequently aminoacylated and rerun on BD-cellulose.

The salt fraction was charged at a very low methionine concentration.

(0.004 nmoles of methionine per normal assay).

The ethanol fraction was charged at a higher methionine concentration.

(2.0 nmoles of methionine per normal assay).

Observed distribution				Expected distribution **		
peak I	peak II	peak III		peak I	peak II	peak III
1%	93%	6%	SALT	2%	70%	28%
4	—	96%	ETHANOL	2%	37%	61%

* 1.25 A_{260} units of tRNA and 5 nmoles of methionine used for charging.

** expected results from table 14 (page 62), for unfractionated tRNA.

3 Properties of H.cutirubrum $tRNA^{met}$ species

11 Heterologous interactions

It had been shown that, although approx. 78% of H.cutirubrum $tRNA^{met}$ was recognised by an E.coli synthetase, when charged heterologously the two species were not resolved on BD-cellulose under conditions suitable for the resolution of homologously charged species (B.N.White, 1970). The availability of H.cutirubrum tRNA of high yield and purity again allowed a more qualitative approach to this heterologous activity and therefore attempts were made to resolve this anomalous chromatographic behaviour.

The acylation of H.cutirubrum and E.coli $tRNA^{met}$ both homologously and heterologously is shown in figure 11. Considerable interaction for the $tRNA^{met}$ of both organisms was indicated; E.coli $tRNA^{met}$ was acylated in the H.cutirubrum system to approx. 60% of the homologous acylation and H.cutirubrum $tRNA^{met}$ was acylated in the E.coli system to approx. 80% of the homologous acylation. The behaviour of H.cutirubrum met-tRNA, after acylation in an E.coli system, on BD-cellulose is shown in figure 12a. An excellent resolution was obtained but it still differed from an homologously charged met-tRNA chromatogram, cf.figure 8a (page 61) noticeably in the absence of the salt species (peak II). Approximately 88% of the met-tRNA was eluted with ethanol whilst the remainder (peak A) eluted in a position between those of peaks I and II found with homologously charged species. The possibility that the ethanol material contained a mixture of peaks II and III seemed unlikely from the result in figure 12b where rechromatography at a high NaCl concentration still resulted in all the cpm. eluting with the ethanol purge.

Figure 11

Homologous and heterologous charging of H.cutirubrum and E.coli tRNA^{not} species

a. H.cutirubrum charging system

Normal charging system (table 4, page 22).

6.3 A_{260} units of H.cutirubrum tRNA.

6.8 A_{260} units of E.coli tRNA.

0.4 mg. of protein in S-150.

Incubation for 30 minutes at 37°C.

b. E.coli charging system

Normal charging system (table 6, page 27).

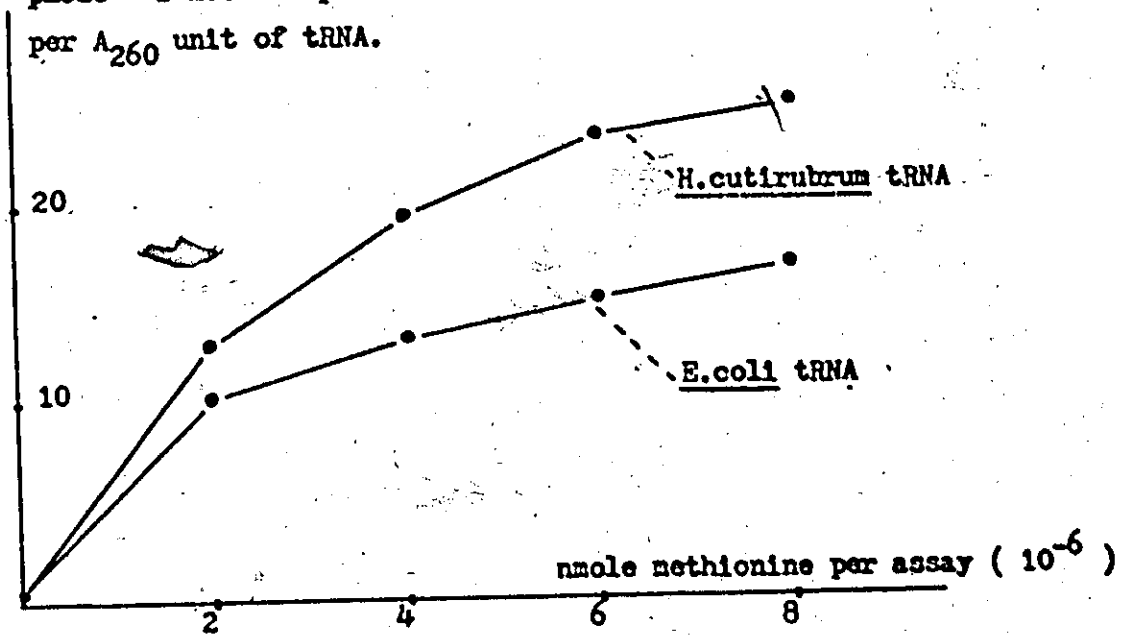
6.4 A_{260} units of H.cutirubrum tRNA.

6.4 A_{260} units of E.coli tRNA.

0.12 mg. of protein in S-150.

Incubation for 30 minutes at 37°C.

a. H.cutirubrum charging system.



b. E.coli charging system.

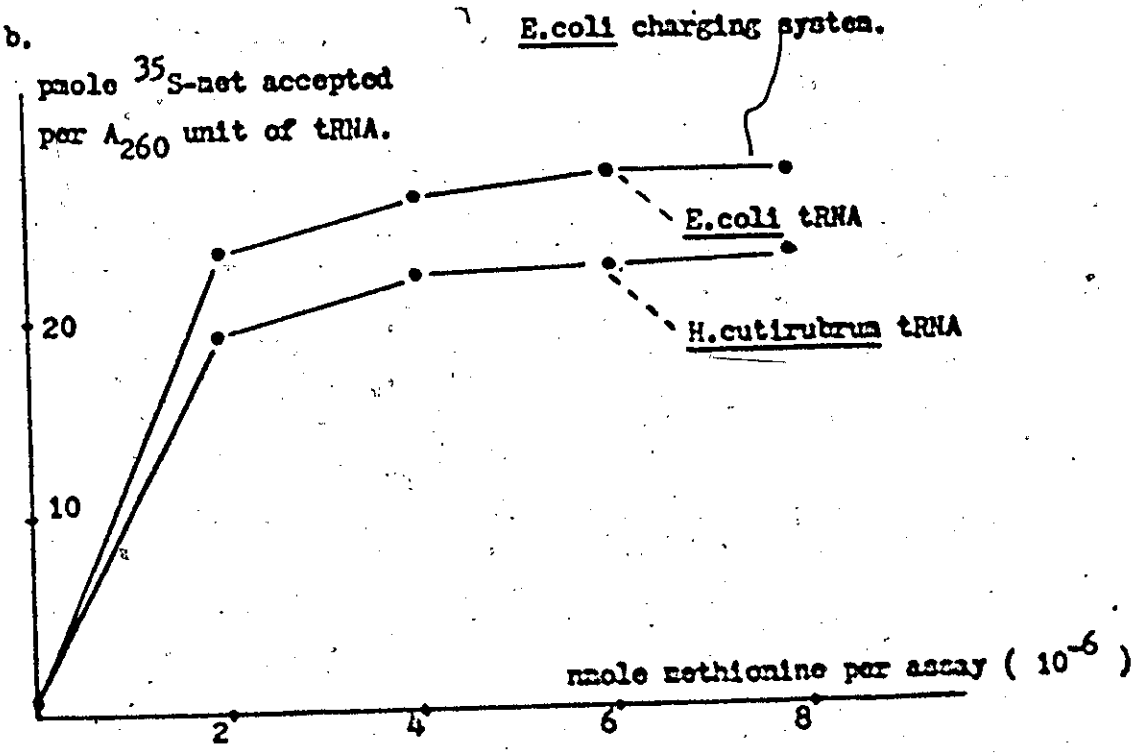


Figure 12

Heterologously charged ^{35}S-met-tRNA on BD-cellulose

a. Unfractionated ^{35}S-met-tRNA

1.5 x 26 cm. column, at 4°C with a flow rate of 36 ml./hr.

Applied : 200 A_{260} units, 2.8 x 10^5 cpm.

Aminoacylation using 6.4 A_{260} units of H.cutirubrum tRNA and 4 nmoles of methionine per normal E.coli assay.

Stepwise elution as shown with all buffers containing 10mM $MgCl_2$, 50mM sodium acetate, pH 4.5, and 5mM 2-mercaptoethanol.

2ml. fractions.

Recovery : complete A_{260} and 80% cpm.

Distribution of cpm. : peak A - 12%, ethanol peak - 88%.

b. Rechromatography of ethanol peak (figure 12a) on BD-cellulose

1.5 x 26 cm. column, at 4°C with a flow rate of 28 ml./hr.

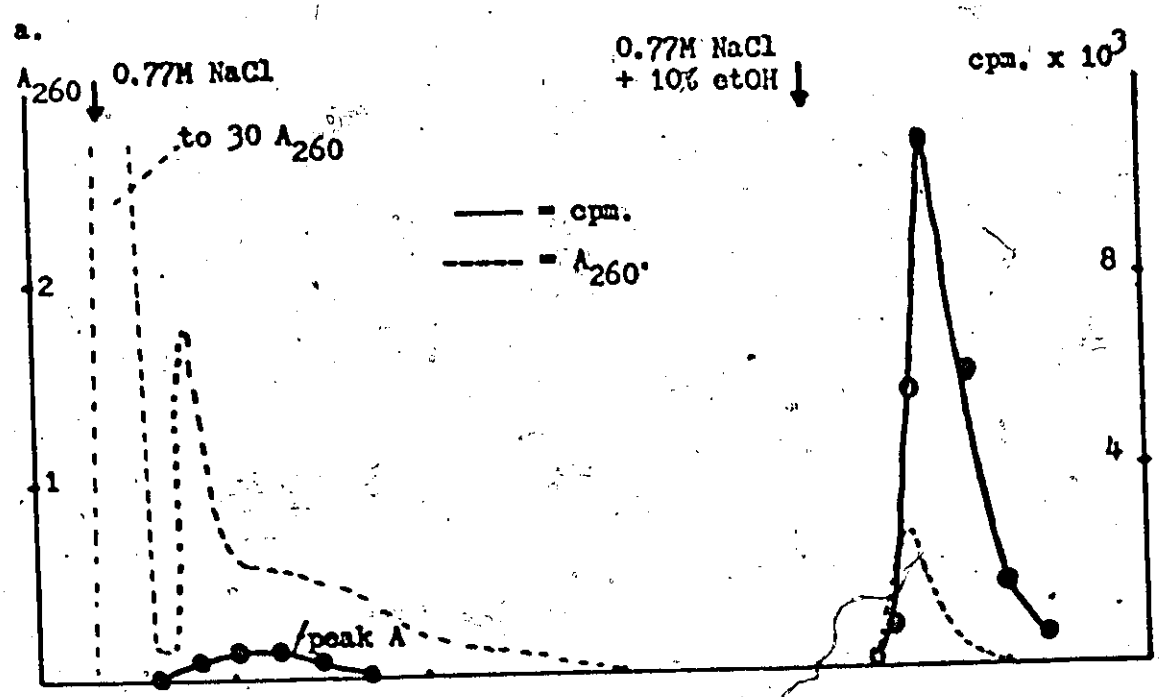
Applied : 6.8 A_{260}, 1.6 x 10^5 cpm. (approx. 135 pmoles/A_{260}).

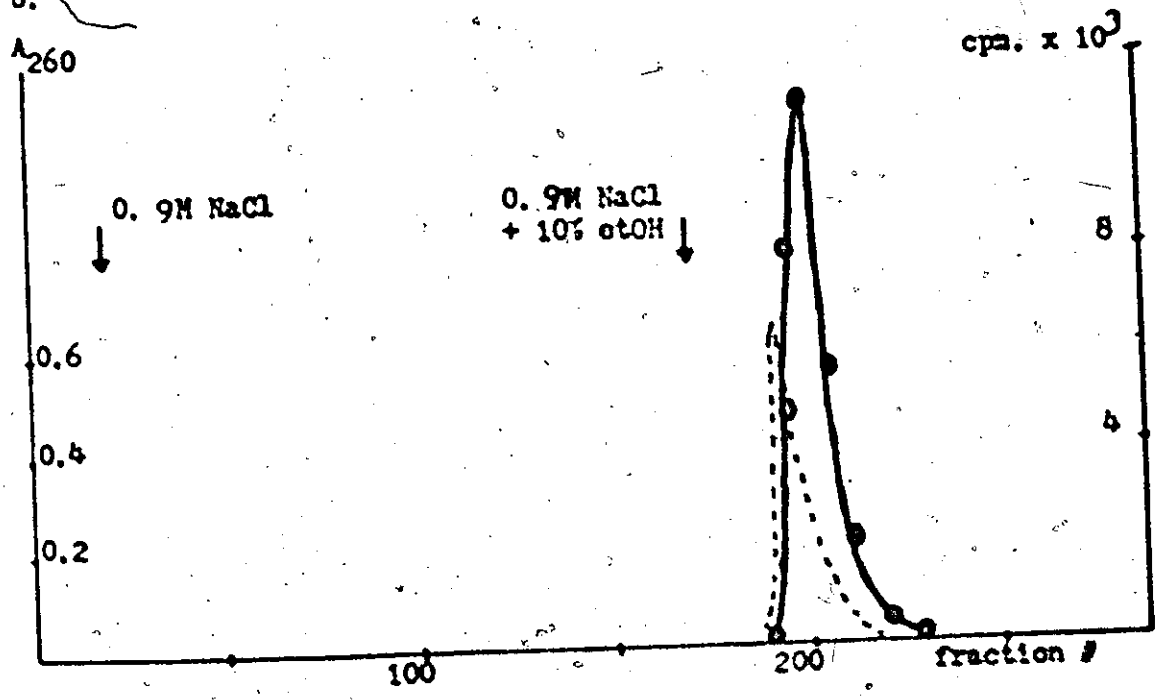
Stepwise elution as shown with all buffers containing 10mM $MgCl_2$, 50mM sodium acetate, pH 4.5, and 5mM 2-mercaptoethanol.

1.5ml. fractions.

Recovery : complete A_{260} and 95% cpm.

Distribution of cpm. : all cpm. eluted with ethanol purge.


a.
A_{260}
0.77M NaCl
to 30 A_{260}
0.77M NaCl
+ 10% etOH
cpm. x 10^3
—— = cpm.
- - - - = A_{260}
2
1
8
4
peak A


b.
A_{260}
cpm. x 10^3
0. 9M NaCl
0. 9M NaCl
+ 10% etOH
0.6
0.4
0.2
8
4
100
200
fraction #

It appeared that the homologously obtained peak II was either not acylated, only slightly acylated and/or chromatographically modified in the E.coli system. To investigate this, homologously charged H.cutirubrum met-tRNA^{met} species were separated on BD-cellulose, deacylated, and then assayed for heterologous acceptance activity. The results for peak II (henceforth the salt species) and peak III (henceforth the ethanol species) are shown in figure 13. Although both species were active homologously (they were used in section 3 iii), only the ethanol species was recognised in the E.coli system and it was acylated to the same extent as, but at a faster rate than, in the homologous H.cutirubrum system (cf. figure 17). It therefore seemed likely that the homologously and heterologously obtained ethanol species (figures 8a & 12a respectively) were identical.

Further support for this conclusion was provided by the chromatogram shown in figure 14. The homologously prepared ethanol species was deacylated, recharged heterologously and rechromatographed; approx. 85% of the met-tRNA reran with the ethanol purge. The remainder eluted as peak A (10%) followed by some tailing (5%) but there was no detectable salt species peak. These heterologous interactions delineated the two H.cutirubrum tRNA^{met} species as a salt species which was not recognised by an E.coli synthetase and an ethanol species which was recognised. This extended the results of B.N.White (1970) who showed that H.cutirubrum tRNA^{met} was formylatable in an E.coli system and that this property resided in the ethanol met-tRNA^{met} species.

The heterologous interactions were extended to include aminoacylation in a eukaryotic system. A yeast charging system (a gift of

Figure 13

Aminoacylation of the salt and ethanol $tRNA^{met}$ species in an E.coli system

Normal E.coli charging system (table 6, page 27).

2.0 A_{260} units of H.cutirubrum salt $tRNA^{met}$.

0.3 A_{260} units of H.cutirubrum ethanol $tRNA^{met}$.

2 moles of methionine in presence of 19 ^{12}C-amino acids.

E-150 used as source of synthetase.

counts without added tRNA have been subtracted.

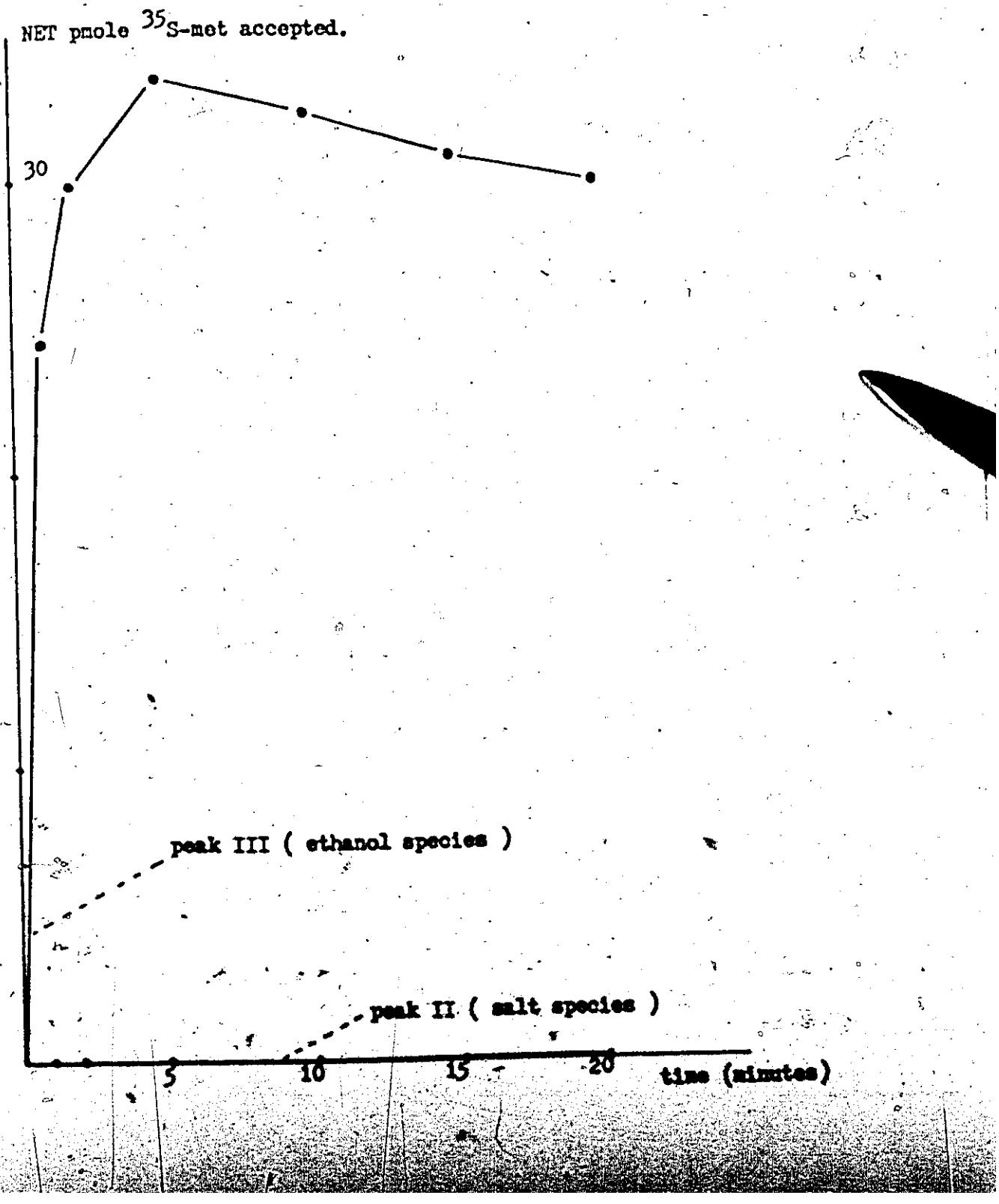

NET pmole 35S-met accepted.
30
peak III (ethanol species)
peak II (salt species)
5
10
15
20
time (minutes)

Figure 14

DEAE-cellulose chromatography of the ethanol species of

M.butyricum met-tRNA acylated by an E.coli synthetase

1.5 x 27 cm. column, at 4°C with a flow rate of 39 ml./hr.

Applied : 20 A_{260} units and 1 x 10^6 cpm.

Aminoacylation in normal E.coli charging system (table 6, page 27)

using very high specific activity ^{35}S-methionine (61,000 mC/mmole).

The tRNA was not dialysed against 5M potassium acetate, pH 4.5, and

the phenol extraction was directly on the incubation mixture.

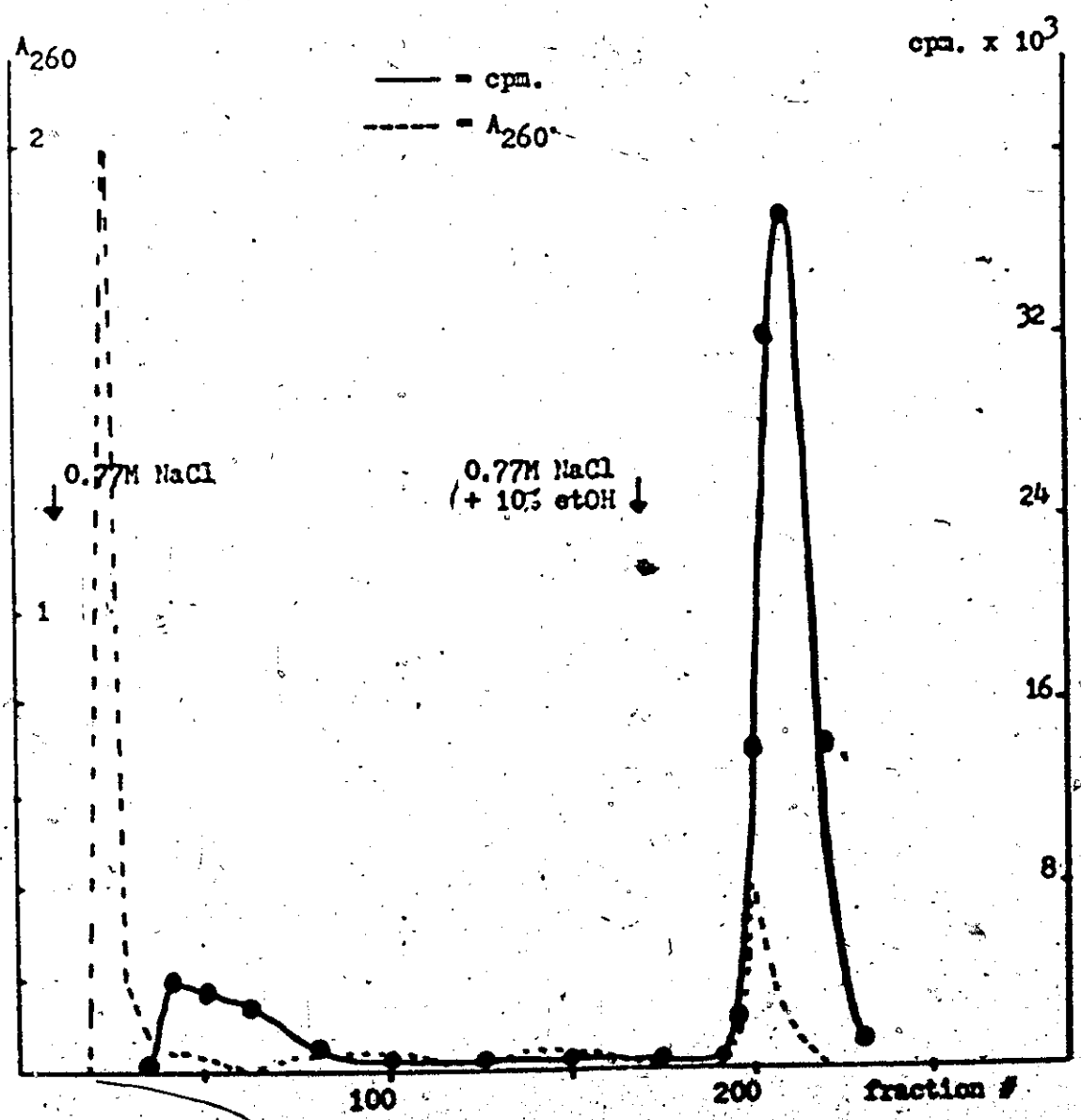
Stepwise elution as shown with all buffers containing 10M $MgCl_2$,

5M sodium acetate, pH 4.5, and 5M 2-mercaptoethanol.

5 ml. fractions.

Recovery : complete A_{260} and cpm.

Distribution of cpm. : 95% of cpm. eluted with ethanol purge.


A_{260}
cpm. x 10^3
—— = cpm.
----- = A_{260}
2
1
32
24
16
8
0.77M NaCl
0.77M NaCl
(+ 10% etOH
100
200
fraction #

G.Sen, B.Sc.) was used to determine whether H.cutirubrum $tRNA^{met}$ species were recognised by a eukaryotic synthetase. The result is shown in table 16 where it is evident that both species were recognised. No attempt was made to optimise the extent of acylation but nevertheless, on a qualitative basis, it appeared that whilst the salt species was not recognised by an E.coli synthetase it was recognised by a yeast synthetase and the ethanol species was recognised by synthetases of both organisms.

Table 16

Aminoacylation of H.cutirubrum tRNA in a yeast charging system

pmole of ^{35}S-met/A_{260} of tRNA

tRNA	H.cutirubrum *	yeast **	charging ***
unfractionated	34.5	18.8	55
salt species	46.5	7.4	16
ethanol species	412	317	77

* normal H.cutirubrum charging system (table 4, page 22).

** yeast charging system was a gift of G.Son.

*** shows acylation by the yeast synthetase as a percentage of the homologous acylation.

Both charging systems used 10 nmoles of methionine, 2.84 A_{260} units of unfractionated tRNA, 3.56 A_{260} units of the salt $tRNA^{met}$ species, 0.075 A_{260} units of the ethanol $tRNA^{met}$ species and incubations were at 37°C for 12 minutes.

3 Properties of H.cutirubrum $tRNA^{met}$ species

111 Homologous reactions and 3' terminal structures

Results shown in the previous section indicated considerable heterologous interaction between H.cutirubrum and E.coli $tRNA^{met}$ species. This suggested similarities in structure concerning synthetase and transformylase recognition; two processes which have been thoroughly studied in E.coli. Marcker (1965) showed that formylation occurred subsequent to the attachment of methionine and that the two $tRNA^{met}$ species resulted in two different RNAse-T_1 digest fragments from their 3' ends. Methionine synthetase has been purified and its properties documented, notably by Bruton & Hartley (1968), Cassio et al (1968) and Lanquet et al, (1972). It was therefore considered worthwhile to look at the homologous acylation of H.cutirubrum $tRNA^{met}$ species and also to undertake a limited RNAse-T_1 digest. This served not only to provide information concerning their structure and behaviour but also in a comparative sense to possibly provide evidence concerning their function.

Ribonuclease T_1 catalyses the cleavage of internucleotide linkages adjacent to a 3'-guanylate residue and thus the composition of an amino acid labelled T_1 digest fragment is strictly dependent on the position of the first guanine residue from the 3' terminal. With E.coli met-tRNA, two such fragments were obtained, met - A-C-C-A-C-C corresponding to the propagator and met-A-C-C-A-A-C corresponding to the initiator. By virtue of the single base difference they were separated by electrophoresis at pH 3.5, the propagator fragment moving towards the cathode and the initiator fragment to the anode (Marcker, 1965).

Both undigested species of H.cutirubrum met-tRNA gave rise to

three components when electrophoresed at pH 3.5, as indicated in figure 15. The large spread of material (1 - 10cm.) towards the anode was considered to be the intact met-tRNA. The peak at 3 - 4 cm. towards the cathode, which ran with and slightly behind a methionine marker, was considered as free ^{35}S-methionine and/or its sulphone. The material at 15 - 19 cm. towards the cathode, which co-electrophoresed with a puromycin marker, was of unknown composition but was at the expected position of methionyladenosine (cf. figure 20b).

RNAse-T_1 digests of both species produced the electrophoretograms shown in figure 16. They differed from those of the intact species notably in the elimination of the met-tRNA, which strongly suggested adequate T_1 activity. Material was still observed to co-electrophorese with puromycin but its proportion was not affected by T_1 digestion. The salt species produced one major peak (1-2 cm. towards the cathode) whilst the ethanol species gave 2 sharp peaks, at 1-2 cm. towards the anode and 1-2 cm. towards the cathode. Overall results comparing electrophoresis before and after T_1 digestion are shown in table 17.

The proportion of ethanol species considered as free ^{35}S-methionine in figure 15b was unchanged after T_1 digestion. If this lack of deacylation similarly applied to the salt species, then the increased material migrating to 1-2 cm. towards the cathode in figure 16a could represent a met-oligonucleotide fragment. If this assumption were correct, although unfortunately no work was undertaken to characterise this material, then the H.cutirubrum $tRNA^{met}$ species would closely resemble the E.coli species as regards their 3' terminal T_1 digest fragments. The ethanol species, the formylatable species, possessed a met-

Figure 15

Electrophoresis of undigested ^{35}S-met-tRNA species

Electrophoresis was carried out as previously described (page 31). Methionine and puromycin were used as markers (see opposite).

Salt species

Applied : approx. 1700 cpm.

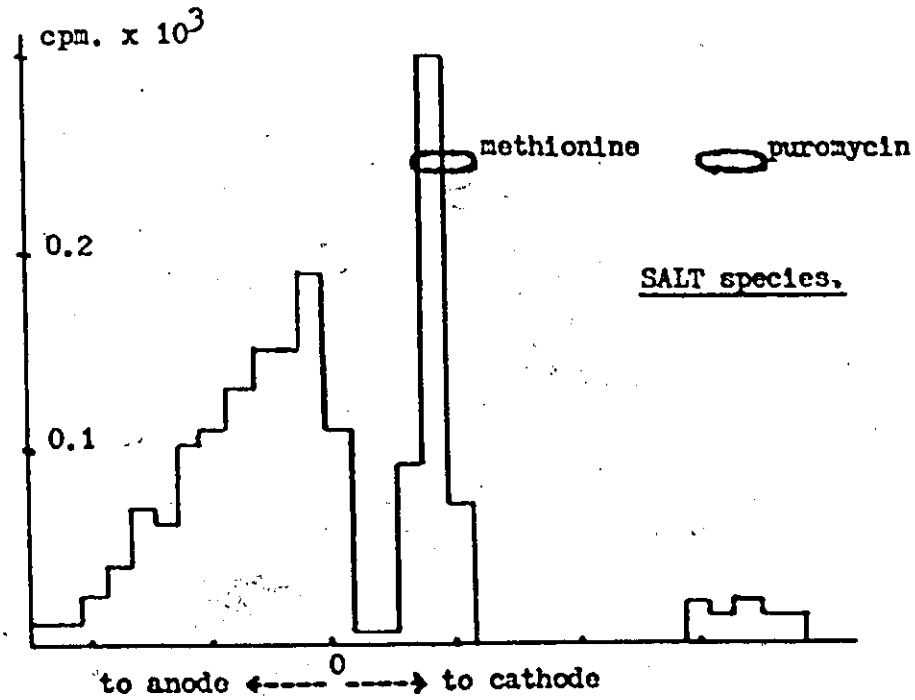
Electrophoresis : 0.5% pyridine - 5% acetic acid, pH 3.5.
Temp. 10°C., 50 V/cm., 100 minutes.

Ethanol species

Applied : approx. 2000 cpm.

Electrophoresis : same as above.

a.


cpm. x 10^3
methionine
puromycin
0.2
SALT species.
0.1
0
to anode ←---- ----→ to cathode

b.

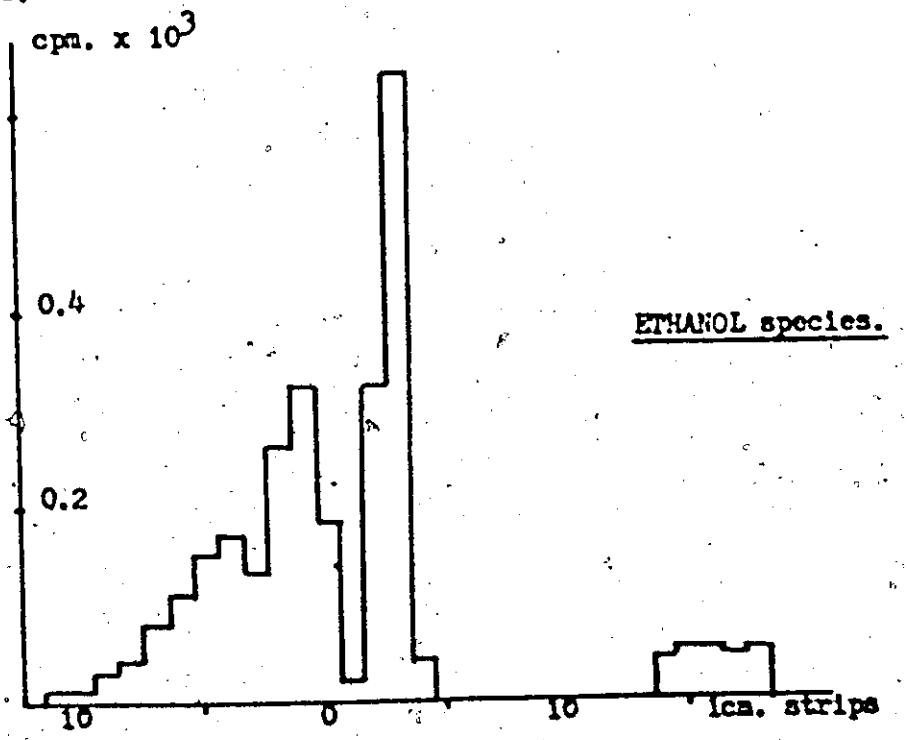

cpm. x 10^3
0.4
ETHANOL species.
0.2
10
0
10
1cm. strips

Figure 1

Electrophoresis of RNAse-T_1 digests of ^{35}S-met-tRNA species

RNAse-T_1 digestion was carried out according to Marcker (1965).

Electrophoresis was as previously described (page 31).

Methionine and puromycin were used as markers (see opposite).

a. Salt species

Applied : approx. 8 x 10^3 cpm.

Electrophoresis : 0.5% pyridine - 5% acetic acid, pH 3.5.

Temp. 10°C. 50 V/cm., 100 minutes.

b. Ethanol species

Applied : approx. 1.4 x 10^4 cpm.

Electrophoresis : same as above.

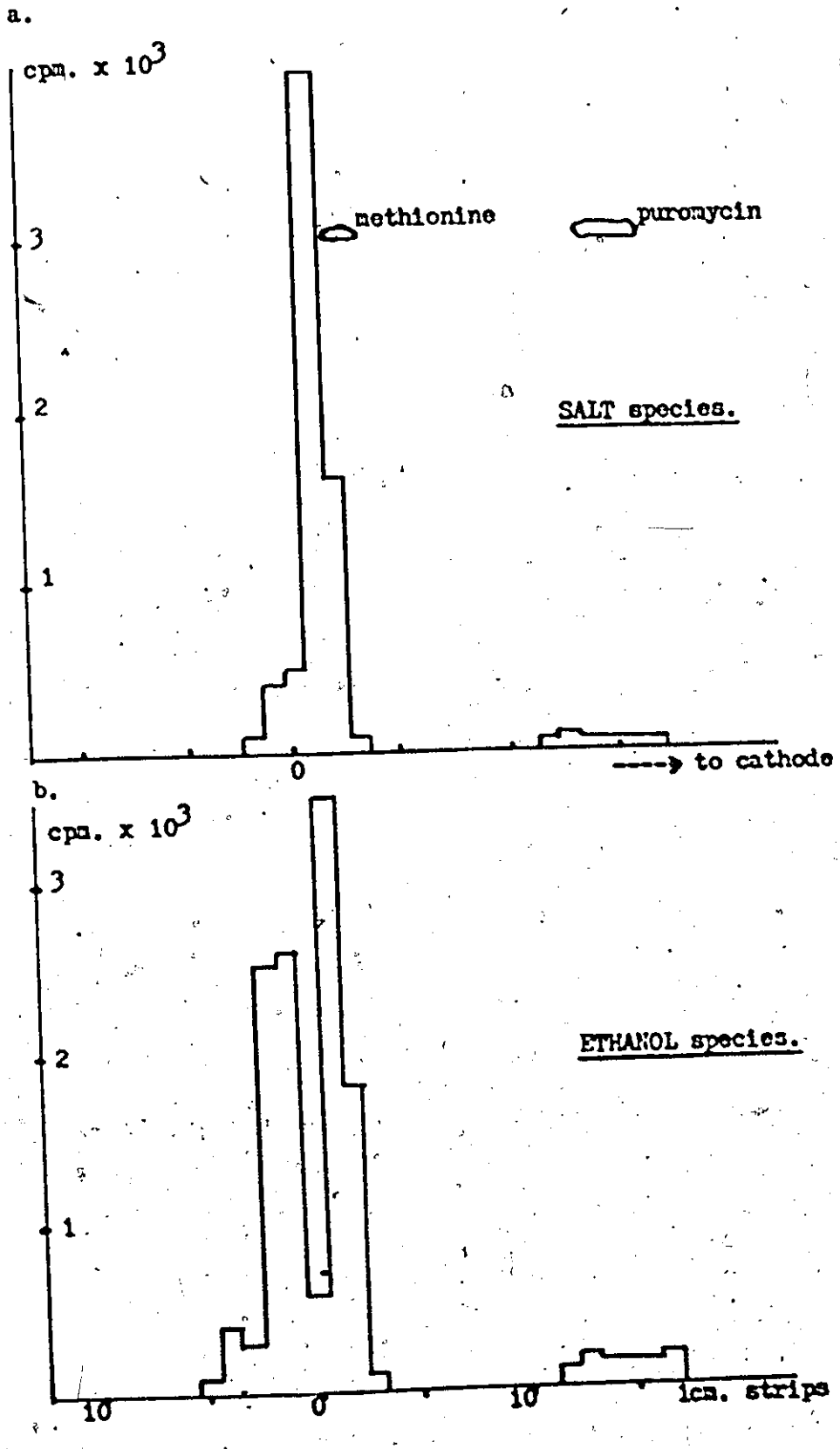

a.
cpm. x 10^3
3
2
1
methionine
puromycin
SALT species.
0
----> to cathode
b.
cpm. x 10^3
3
2
1
ETHANOL species.
10
0
10
1cm. strips

Table 17

RNAse-T_1 digestion of H.cutirubrum ^{35}S-met-tRNA species

^{35}S-met-tRNA	% distribution on Whatman 3MM paper		
	migration to anode	migration to cathode (1-2 cm.)	migration to cathode (17 cm.)
Salt species			
undigested	68%	28%	4%
T_1 digest	15%	80%	5%
Ethanol species			
undigested	54%	38%	8%
T_1 digest	51%	41%	8%

The results for this table have been obtained from figures 15 and 16. Herbert, Smith & Wilson (1964) have shown that ribonuclease T_1 digestion of aa-tRNA can be performed under conditions where the hydrolysis of the aa-tRNA is negligible, these conditions were followed in the experiment described in figure 16.

oligonucleotide fragment which was negatively charged at pH 3.5 whilst the salt species tentatively possessed a not-oligonucleotide fragment positively charged.

Thus RNAse-T_1 digestion provided information on three matters concerning H.cutirubrum $tRNA^{met}$. Firstly it suggested that the two species differed at their 3' ends in a manner resembling the difference between E.coli $tRNA^{met}$ species. Secondly it showed little cross contamination of species resulting from chromatography on BD-cellulose. Thirdly it indicated the presence, albeit after storage at -20° in 5mM NAc, pH 4.5 with repeated freezing and thawing, of an unknown radioactive contaminant, positively charged at pH 3.5 which accounted for 4% of the salt species and 8% of the ethanol species.

Homologous acylation studies were originally undertaken as a control for confirmation of the new proportion of species obtained in figure 3a (page 61) but they also served as a means of comparison with the reported relationships of E.coli $tRNA^{met}$ species and their synthetase.

The rates of acylation for the separated species were obtained under equivalent conditions and are shown in figure 17. The salt species possessed the greater rate both at low and high methionine concentrations. This concurred with table 14 (page 62) which had indicated that the proportion of species obtained from BD-cellulose was consequent on the extent of acylation, which was dependent upon the methionine concentration used for charging. At low methionine concentration, the salt species, by virtue of its faster rate, appeared the major species, but as the methionine increased, providing a greater extent of acylation, chromatography revealed a truer distribution, one in which the ethanol $tRNA^{met}$

Figure 17

Aminoacylation of the salt and ethanol $tRNA^{met}$ species in an H.cutirubrum system

The reaction mixture (table 4, page 22) was modified to a final nominal volume of 0.1 ml. The final salt concentrations of table 4 were retained.

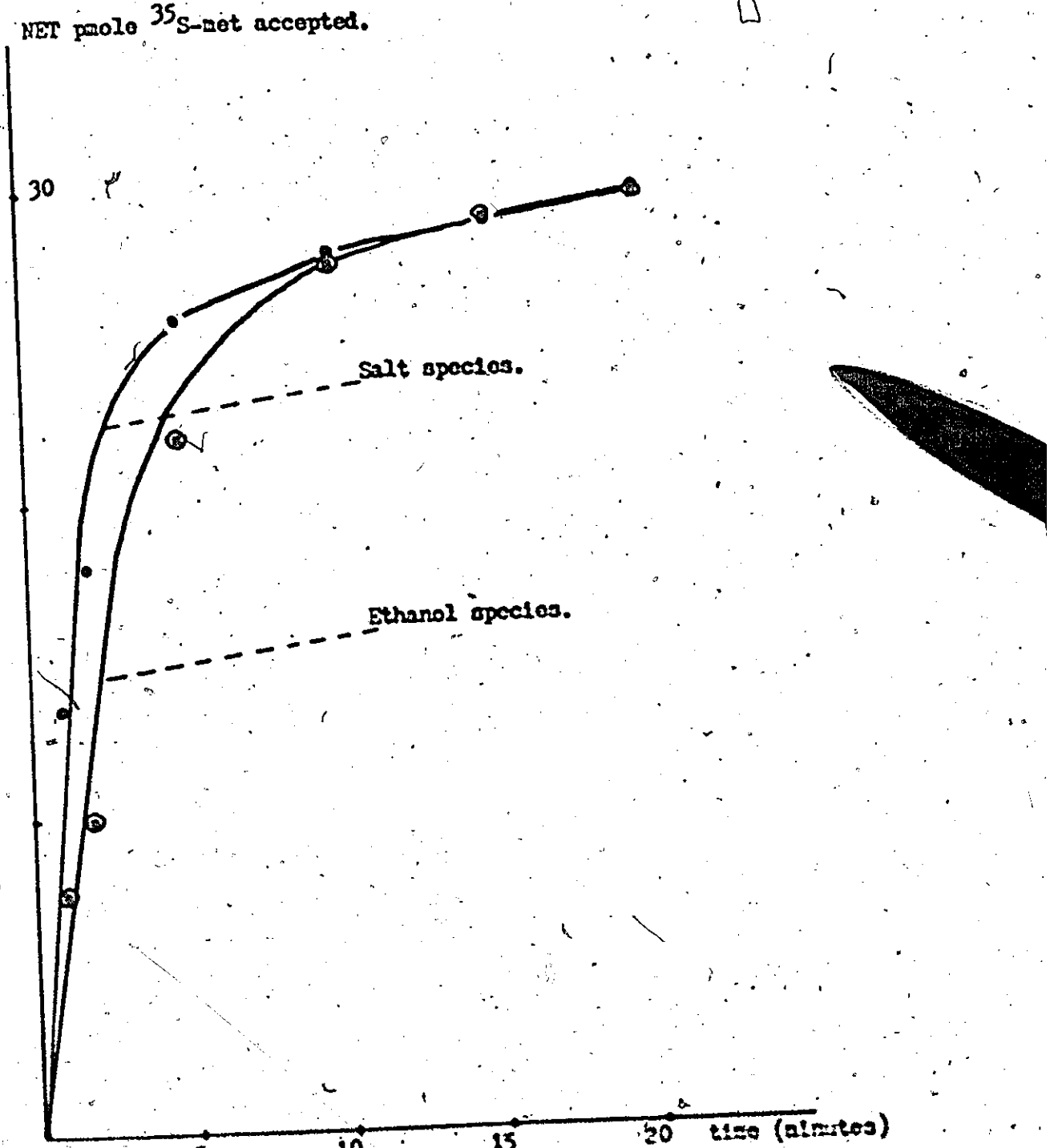
2.00 A_{260} units of H.cutirubrum salt species.

0.29 A_{260} units of H.cutirubrum ethanol species.

8 nmoles of methionine in presence of 19 ^{12}C-amino acids.

S-150 used as source of synthetase.

counts without added tRNA have been subtracted.


NET pmole 35S-met accepted.
30
Salt species.
Ethanol species.
5
10
15
20
time (minutes)

was the major species.

Determination of the K_m values of the $tRNA^{met}$ species provided an explanation for the faster rate of the salt species. The apparent K_m of the salt species was approx. 2×10^{-6} M whereas that for the ethanol species was approx. 6×10^{-6} M (figure 18). These values were of the same order of magnitude as those described for E.coli by Cassio et al (1968), 1.2×10^{-6}M for $tRNA_m^{met}$ and 1.7×10^{-6}M for $tRNA_f^{met}$. They differed from those reported by Bruton and Hartley (1968), 3×10^{-7}M for $tRNA_m^{met}$ and 5×10^{-7}M for $tRNA_f^{met}$ but in both cases they compared favourably with the observation that the propagator species possessed the lower K_m value.

Although the apparent K_m value of leucine for halophilic acylation had been reported to be similar to that for an E.coli synthetase, (Griffiths and Bayley, 1969), later work showed that it was not a typical amino acid and that the concentration of amino acids was a major control over the extent of acylation. It was suggested that the apparent K_m of the synthetase for other amino acids was higher in the halophilic system (B.N.White, 1970). In view of the control exerted by methionine on the extent of acylation, table 14 (page 62), and the difference in activity using high specific activity methionine in the homologous and heterologous systems, figure 11 (page 72), it was considered worthwhile to determine the K_m value of methionine.

B.N.White (1970) had indicated that the presence of 1.4M NaCl during aminoacylation was stimulatory for the majority of amino acids but inhibitory for methionine. Figure 19 provides Lineweaver-Burke plots for the acylation of unfractionated $tRNA^{met}$ with varying concen-

Figure 18

Determination of the K_m values for the salt and ethanol species

Modified H.cutirubrum charging system (see figure 17, page 90).

After a prior incubation to dissolve the added dry KCl, complete reaction mixtures were incubated for 2 minutes at 37°C.

Each incubation contained 500 nmoles of ATP and 10 nmoles of methionine.

The 19 ^{12}C-amino acids were omitted.

Estimation of the pmole of $tRNA^{met}$ added was made on the basis of the maximum acylation achieved by the two species with the component concentrations as described : salt species - 16.5 pmole $tRNA^{met}/A_{260}$.

ethanol species - 110 pmole $tRNA^{met}/A_{260}$.

S-150 was used as a source of synthetase.

Results without added tRNA have been subtracted.

V = pmole ^{35}S-methionine in cold 5% TCA precipitate.

S = pmole $tRNA^{met}$ per 0.1ml. nominal reaction volume (cf.table 2, page 16).

Apparent K_m for the salt species = 2.0×10^{-6}M.

Apparent K_m for the etOH species = 6.0×10^{-6}M.

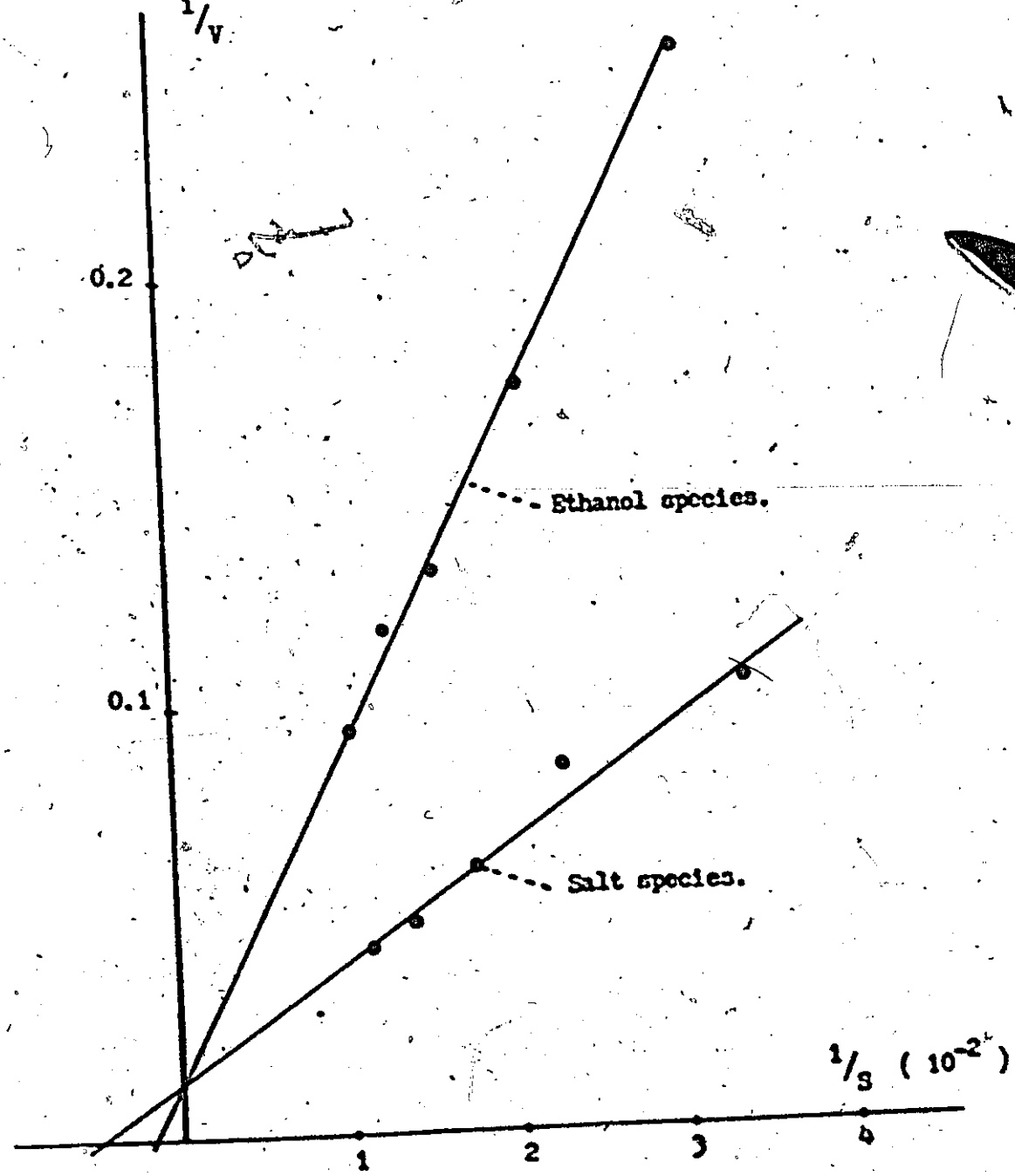

1/V
0.2
0.1
Ethanol species.
Salt species.
1/S (10^-2)
1
2
3
4

Figure 19

Determination of K_m value for methionine

Normal H.cutirubrum charging system (table 4, page 22).

Assays were carried out as for figure 18 (page 93).

Each incubation contained 500 nmole of ATP. 4 A_{260} units of unfractionated H.cutirubrum tRNA (35 pmole $tRNA^{met}/A_{260}$) and the 19 ^{12}C-amino acids were omitted.

Methionine was added at a concentration of 2×10^{-4}M and a specific activity of 168 mC/mmole.

0.2 A_{260} units of peak 1 from a G-75 Sephadex column of a 100% $(NH_4)_2SO_4$ precipitate of an S-150 were used as a source of synthetase.

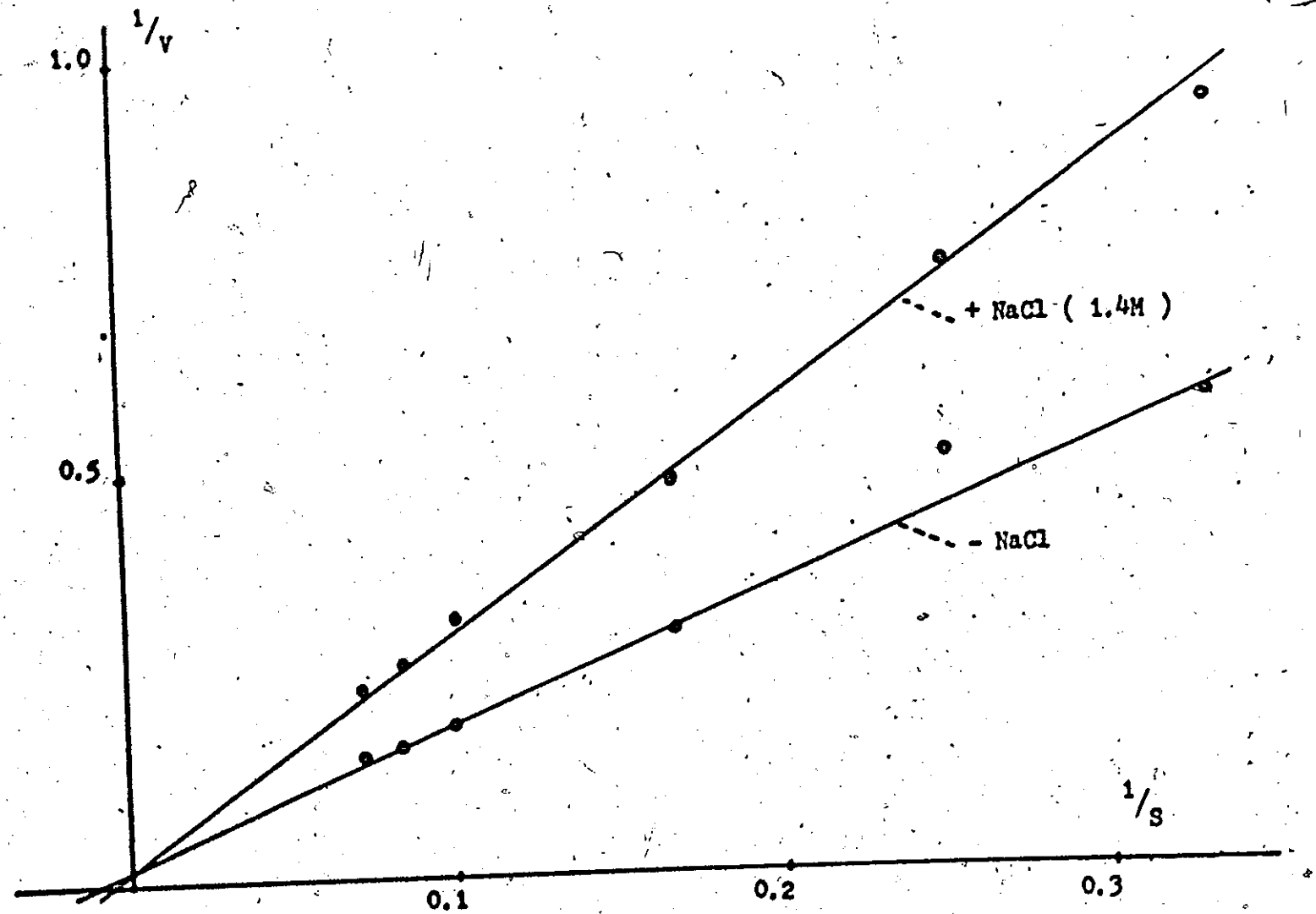
Y = cpm. $\times 10^{-3}$ in cold 5% TCA precipitate.

S = microlitres of methionine per 0.225ml. nominal reaction volume (cf. table 2, page 16).

Apparent K_m in absence of NaCl = 4.5×10^{-5}M.

Apparent K_m in presence of 1.4M NaCl = 1.2×10^{-4}M.

N.B. the concentration of $tRNA^{met}$ used in this experiment was lower than the K_m values determined in figure 18.


1/V
1.0
0.5
+ NaCl (1.4M)
- NaCl
1/S
0.1
0.2
0.3

trations of methionine both in the presence and absence of 1.4M NaCl. The apparent K_m for methionine under normal conditions i.e. without NaCl was calculated at approximately 4.5×10^{-5}M. This value was greater than that determined for leucine, 4.2×10^{-6}M, but it compared favourably with 2×10^{-5}M for E.coli methionine synthetase as reported by Heinrikson & Hartley (1967). The effect of NaCl appeared to be an increase in the apparent K_m to approximately 1.2×10^{-4}M whilst no change was observed in the V_{max}. These kinetics were suggestive of competitive inhibition (Dixon & Webb, 1964) between the Na^+ ions and the enzyme.

This brief acylation study compared the K_m values of methionine and $tRNA^{met}$ species in the H.cutirubrum system favourably with the E.coli system. It supported and provided an explanation for the observed distribution of species and finally it indicated a difference in recognition for the salt and ethanol species by E.coli synthetase.

4 In vivo initiation studies

Whilst attempting to achieve resolution of $tRNA^{met}$ species it was not possible to use *in vitro* assay systems and therefore an alternative *in vivo* approach was selected. This was based on experiments described by Bachmayer & Kreil (1968) and although it would not differentiate between $tRNA^{met}$ species it could indicate the role played by methionine.

The absence of formyl-methionine in *H.cutirubrum* grown under normal laboratory conditions was reported previously by White & Bayley (1972c). Their findings were confirmed in a similar experiment described in figure 20. This absence did not eliminate methionine as an initiator since the same authors indicated that one $tRNA^{met}$ species was formylatable by an *E.coli* transformylase and suggested that initiation might involve an unblocked methionine. A means for testing this possibility involved the use of puromycin; an antibiotic which acts as an analogue of aminoacyl-tRNAs and inhibits protein synthesis by causing premature termination of growing polypeptide chains (Yarmolinsky & Haba, 1959).

Bachmayer & Kreil (1968) investigated initiation in a number of prokaryotic systems, four bacteria and one blue-green alga. They treated *in vivo* cultures with puromycin and then characterised an initiator amino-acyl-puromycin complex, by its extraction into ethyl acetate and its identification by high voltage electrophoresis. The rationale for this experiment was that puromycin terminated polypeptide synthesis immediately after initiation. The application of this technique to an *in vivo* culture of *H.cutirubrum* is shown in figure 21.

Puromycin's effect was evident as two small peaks, A & B migrating

Figure 20

Absence of formylmethionine in H.cutirubrum

24 hour H.cutirubrum cells, grown in 100ml. of complex medium, were harvested and resuspended in 0.5ml. of synthetic medium (Ochishi et al, 1964) devoid of methionine. ^{12}C- and ^{35}S-methionine (approx. 70 mcales, specific activity 800 mC/mmole) were then added and the culture incubated at 37°C for 45 minutes. Cells were harvested, washed, and lysed by homogenization in 5mM KAc, pH 4.5 and 2% cutscum. Nucleic acids were phenol extracted and precipitated with ethanol. The aa-tRNA was then either digested with RNAse or deacylated by alkaline hydrolysis.

a. Electrophoresis of products of deacylation

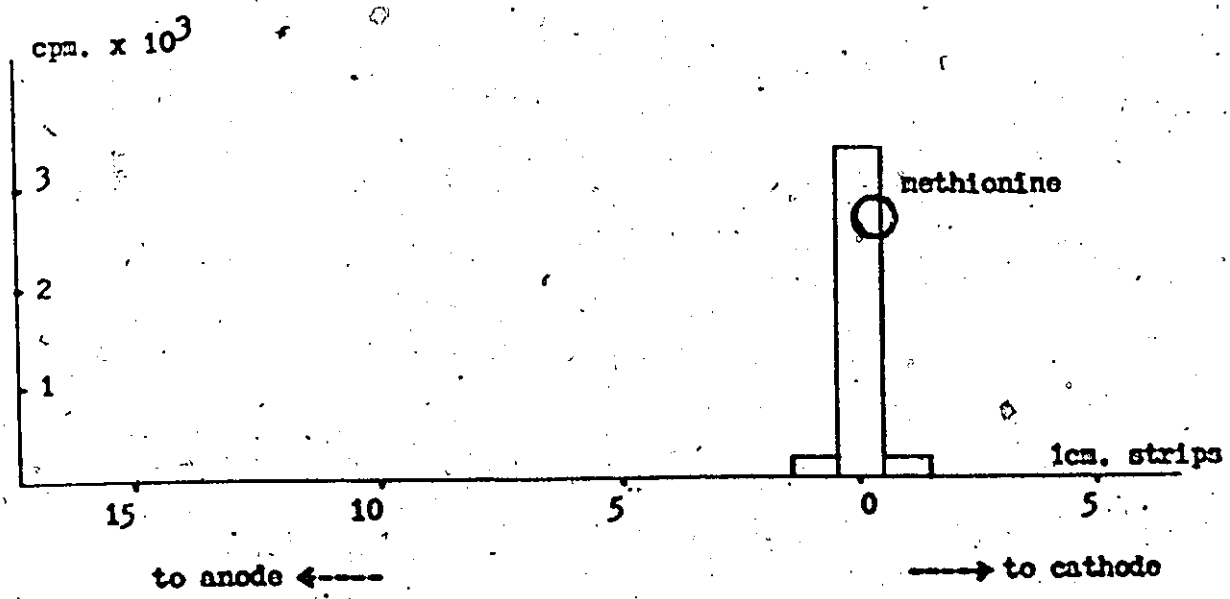
Aminoacids, discharged by alkali (0.1N NaOH for 30min. at 37°C), were dried from the ethanolic supernatant remaining after precipitation of nucleic acid. They were then electrophoresed as previously described (page 31). Approx. 4.5×10^3 cpm. were applied to the Whatman 3MM paper.

b. Electrophoresis of products of RNAse digestion

Nucleic acid in 0.5ml. of water was digested with T1RNAse (0.01mg.) and pancreatic RNAse (0.01mg.) at room temperature for 5 minutes. This solution was then used directly for electrophoresis. Approx. 4×10^3 cpm. were applied to the Whatman 3MM paper.

Methionine and puromycin were run as markers.

a.


cpm. x 10^3
3
2
1
methionine
1cm. strips
15
10
5
0
5
to anode
to cathode

b.

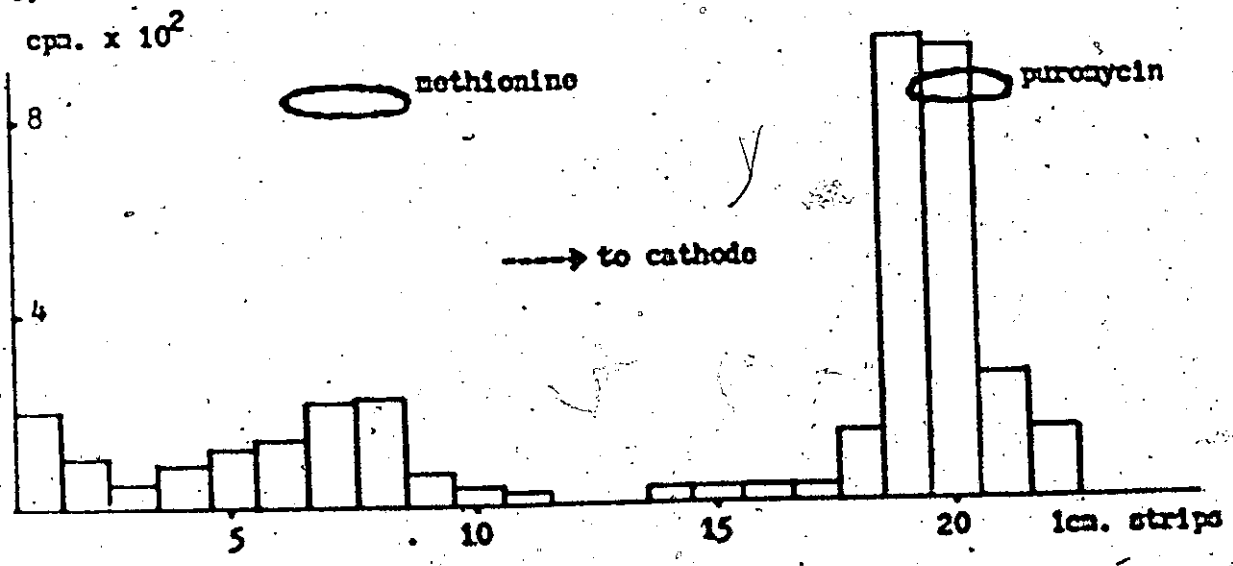

cpm. x 10^2
8
4
methionine
puromycin
to cathode
5
10
15
20
1cm. strips

Figure 21

Formation of methionyl - puromycin in vivo

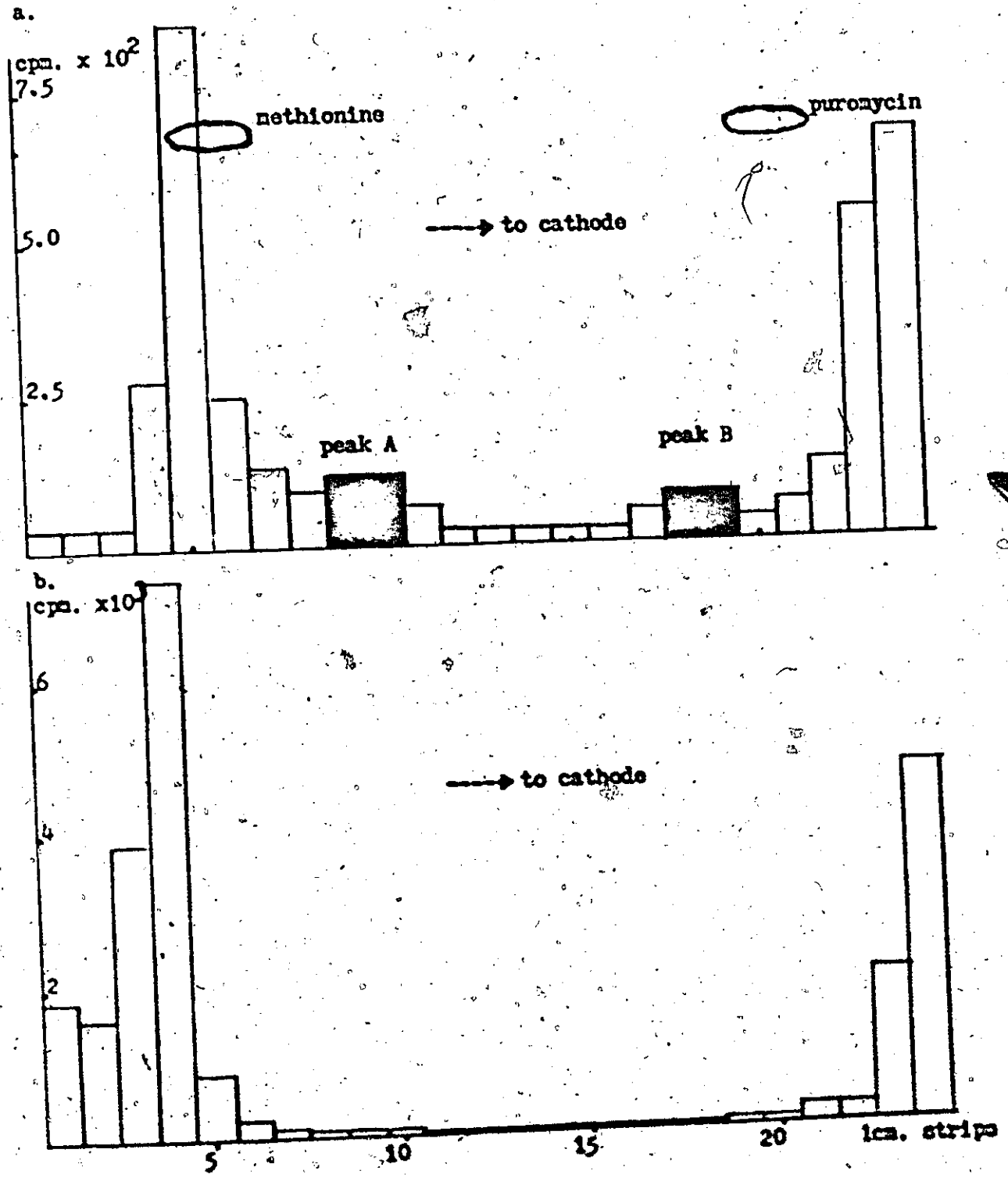
24 hour H.cutirubrum cells, grown in 100ml. of complex medium, were harvested and resuspended in 0.4ml. of synthetic medium (Onishi et al, 1964) devoid of methionine. The culture was then treated as below.

a. Cells treated with puromycin

Puromycin and ^{12}C- and ^{35}S-methionine were added to the culture to final concentrations of 9mM and 0.1mM (sp. act. approx. 300 mC/mmole) respectively. After incubation at 37°C for 1 hour an ethyl acetate extraction was carried out directly on the culture. The pH of this mixture was maintained at approximately 8.5 by the addition of 1M KOH, and 0.1ml. of 2% cutscum was added to facilitate cellular disruption. The ethyl acetate phase was obtained by centrifugation and dried under vacuum at room temperature. This material was electrophoresed at pH 3.5 as described previously (page 31). Approximately 7.5×10^3 cpm. were applied to the Whatman 3MM paper.

b. Control culture without added puromycin

The culture was treated as described above except that NaCl was added in place of puromycin. The specific activity of ^{35}S-methionine was approx. 800 mC/mmole. Approximately 4.5×10^4 cpm. were applied to the Whatman 3MM paper.


a.
cpm. x 10^2
7.5
5.0
2.5
methionine
puromycin
to cathode
peak A
peak B
b.
cpm. x10
6
4
2
to cathode
5
10
15
20
1cm. strips

to the reported positions of F-met-puromycin and met-puromycin (Leder & Buraszyn, 1966) respectively. Peak A accounted for approx. 0.03% and peak B approx. 0.015% of the original ^{35}S-methionine added to the culture. These values were very low when compared to those of Bachmayer & Kreil (1968) who had reported a recovery of 0.02 - 0.8%, which related to the amount of met-puromycin present after deformylation of F-met-puromycin and did not include any met-puromycin present in the original ethyl acetate extraction. Although the results with H.cutirubrum appeared to rule out methionine as an initiator on a quantitative basis, peaks A and B were nevertheless consistent features of the effect of puromycin and attempts were made to identify them.

Peaks A and B were quantitatively adsorbed to 'Norite' (approx.80%) both before and after dilute NH_4OH (pH 10.5) treatment. This was intended to indicate the linkage of methionine to an aromatic compound, (Bretscher & Marcker, 1966), but unfortunately methionine itself was adsorbed to the charcoal to approximately the same extent (75%). Therefore no definite indication of an aromatic compound was obtained.

Peak A was not F-met-puromycin, since dilute methanolic HCl, which selectively cleaves formyl groups, did not cause material to migrate to the position of met-puromycin. The material moved instead to a position less basic than methionine, corresponding approximately to methionine sulphone, as shown in figure 22b. Peak A was resistant to dilute alkali, which suggested it was not a compound such as N-blocked-met-adenosine, N-blocked-aa-met-puromycin or met-aa-puromycin, as shown in figure 22c. Its absorbance characteristics in distilled H_2O were min. 246mμ., max. 255mμ., with a slight shoulder at 275mμ.

Figure 22

Attempts at the identification of peak A (figure 21a)

a. Re-electrophoresis of peak A

Peak A (figure 21a) was eluted from the Whatman 3MM paper with ethyl acetate-methanol-water (1:1:1), dried under vacuum at room temperature and re-electrophoresed as described for figure 21. Approximately 700 cpm. was applied to the Whatman 3MM paper.

b. Electrophoresis of peak A after treatment with methanolic HCl

Peak A (figure 21a), after elution from the Whatman 3MM paper, was treated with 0.2N HCl in methanol (Bachmayer & Kreil, 1968) at 37°C for 3 hours. This procedure should selectively cleave formyl groups. Peak A, approx. 2×10^3 cpm., was then applied to the Whatman 3MM paper and electrophoresed at pH 3.5 as previously described.

c. Electrophoresis of peak A after alkaline hydrolysis

Peak A (figure 21a), after elution from the Whatman 3MM paper, was treated with dilute NH_4OH, pH 10.5, at 37°C for 1 hour (Marcker & Sanger, 1964). This should cleave met-ea or met-adenosine derivatives but should not affect a met-puromycin derivative. Peak A, approx. 600 cpm., was then applied to the Whatman 3MM paper and electrophoresed at pH 3.5 as previously described.

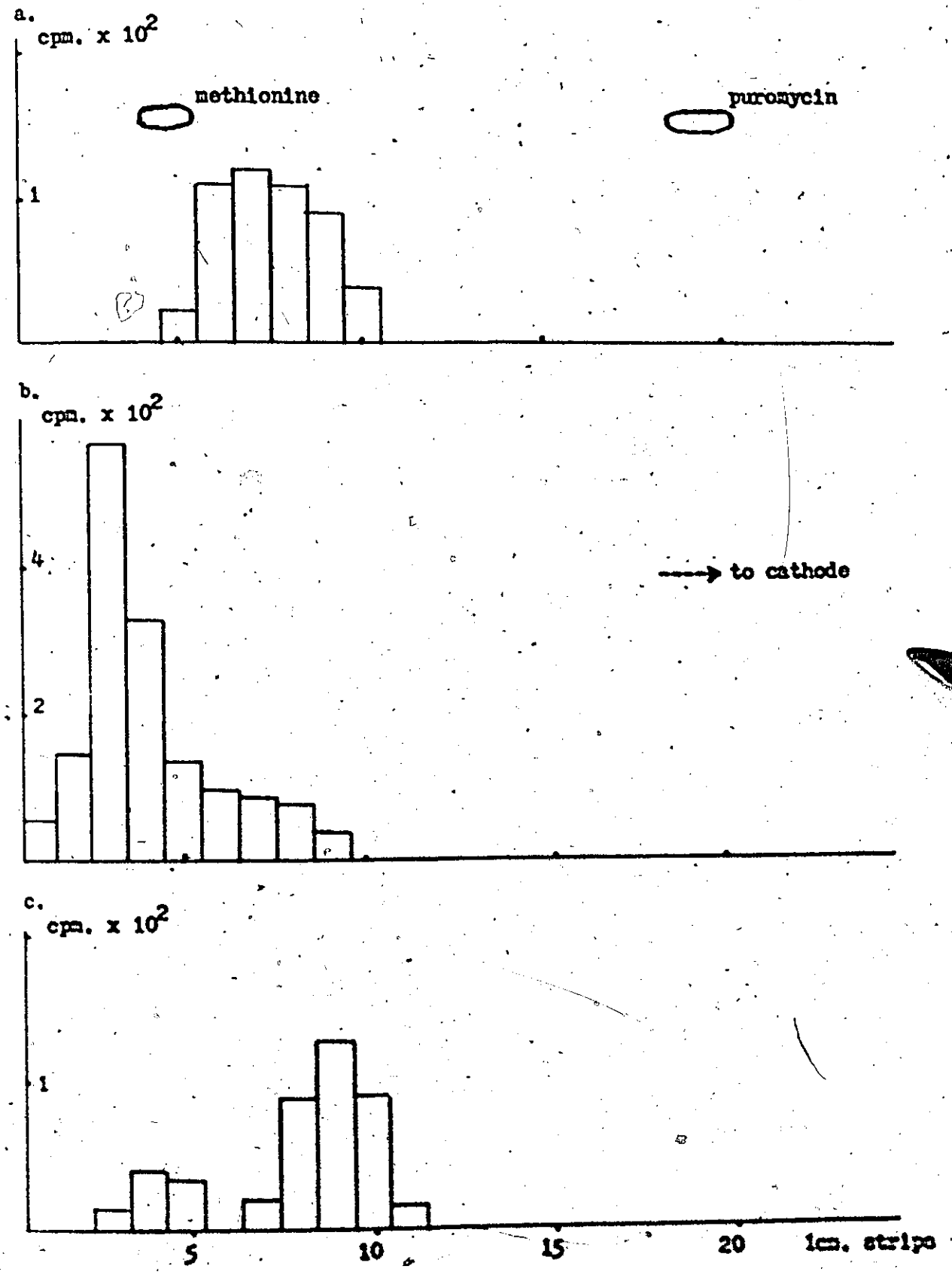

a.
cpm. x 10^2
methionine
puromycin
1
b.
cpm. x 10^2
4
2
to cathode
c.
cpm. x 10^2
1
5
10
15
20
1cm. strips

Figure 23

Attempts at the identification of peak B (figure 21a)

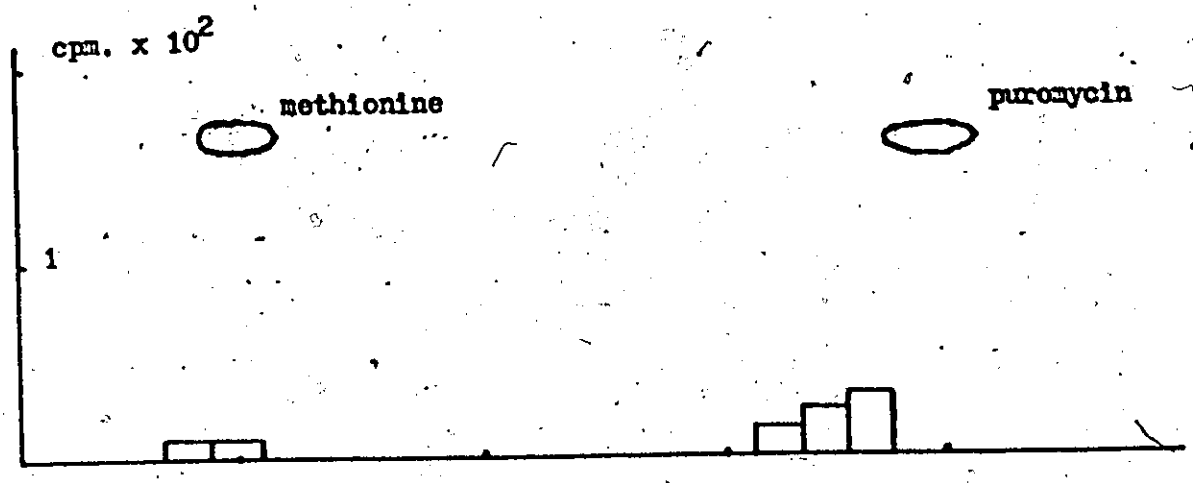
a. Re-electrophoresis of peak B

Peak B (figure 21a) was eluted from the Whatman 3MM paper with ethyl acetate-methanol-water (1:1:1), dried under vacuum at room temperature and re-electrophoresed as described for figure 21. Approximately 100 cpm. were applied to the Whatman 3MM paper.

b. Electrophoresis of peak B after alkaline hydrolysis

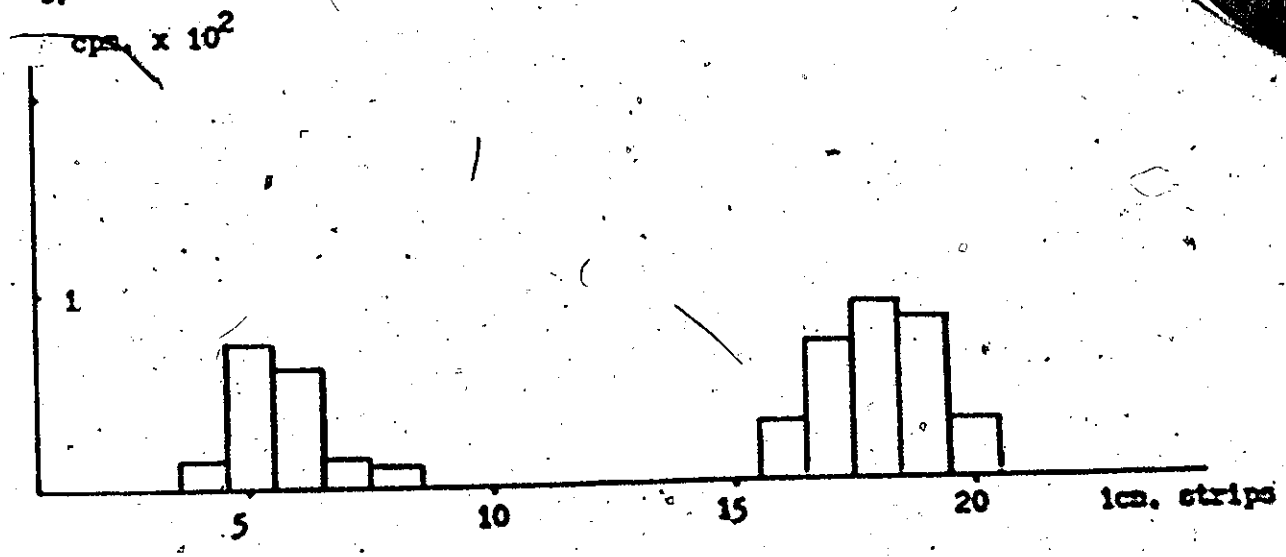
Peak B (figure 21a), after elution from the Whatman 3MM paper, was treated with dilute NH_4OH, pH 10.5, at 37°C for 1 hour. Approximately 500 cpm. were then applied to the Whatman 3MM paper and electrophoresed at pH 3.5 as previously described.

a.



→ to cathode

b.



Peak B was not not-adenosine, as shown by its resistance to dilute alkali in figure 23b. Its absorbance characteristics in distilled H_2O were min. 242, max. 274mm., which were essentially the same as puromycin. However, this similarity was probably due to the presence of free puromycin which was extracted into ethyl acetate and overlapped peak B on the electropheretogram. The poor yield of peaks A and B prevented further analysis and no positive identification was achieved.

The overall results of this investigation did not support the role of methionine as an initiator but neither did they completely disprove the possibility. Peaks A and B remain unidentified and the reasons for their poor yield could be related to the adaptation of the technique for H.cutirubrum or to physiological conditions pertaining to this organism.

5 In vitro initiation studies

1 Codon recognition

In 1966 the genetic code was broken (Crick, 1966a) and although the evidence used to elucidate the codon assignments came mainly from E.coli it seemed likely that the code in other organisms was either identical or very similar to that in E.coli. The designated codon for methionine was AUG but in addition the initiator $tRNA_f^{met}$ possessed the ability to recognise GUG, normally a valine codon, as an initiating signal (Ghosh et al, 1967). It was therefore relevant to determine whether the same relationship existed for H.cutirubrum $tRNA^{met}$ species.

The H.cutirubrum ribosomal binding system, as described by White & Bayley (1972a), was used to determine the response towards the trinucleotide ApUpG and to poly (GU; 1:1). The result, shown in table 18, indicated only a marginal response to both templates. This response was consistent but attempts at significantly increasing it proved unsuccessful. The reason for the poor recognition of poly (GU) was not clear since the same polymer was active in polypeptide synthesis, cf. table 19, and methionine incorporation into polypeptide was stimulated by a random poly (GU) template (Bayley & Griffiths, 1968b). The apparent failure of ApUpG was equally puzzling but to date, a significant response towards a trinucleotide codon has not been demonstrated in the halophilic binding system.

Because of the lack of significant response in the halophilic system, attention was turned to the use of the H.cutirubrum met-tRNAs in an E.coli binding system. Although this step was subject to the proviso of using the tRNA in a foreign ionic environment, it was still considered justified, especially in light of the results of White & Bayley (1972b).

Table 18

Ribosomal binding of H.cutirubrum met-tRNA species in response to ApUpG and poly(GU) in an H.cutirubrum system

mM Mg^{2+}	pmole of ^{35}S-met-tRNA bound to ribosome					
	salt species			ethanol species		
	ApUpG	poly(GU)	-template	ApUpG	poly(GU)	-template
24	0.13	0.16	0.14	0.23	0.27	0.22
40	0.23	0.17	0.14	0.39	0.38	0.30
56	0.22	0.27	0.21	0.47	0.46	0.37
88	0.31	0.39	0.26	0.57	0.60	0.48

Incubation at 37°C for 40 min. in normal binding system (table 5, page 25).

Pre-incubated ribosomes (page 15) : 7.2 A_{260} units.

ApUpG : 0.135 A_{260} units, approx. 3.75 nmoles.

Poly (GU, 1:1) : 1.0 A_{260} unit, 0.05mg.

Salt species : 0.34 A_{260} units, 1590 cpm., approx. 8.85 pmoles.

Ethanol species : 0.09 A_{260} units, 1560 cpm., approx. 8.65 pmoles.

Deacylation of aa-tRNA during binding assay :

Complete reaction mixture (minus ribosomes and template).

Salt species : 82 at 37°C for 40 minutes.

Ethanol species : 82

These authors suggested that the codon-anticodon interactions of aminoacyl-tRNAs from both bacteria were the same in both low and very high salt assay systems.

The response of both species to ApUpG at various magnesium concentrations in the E.coli binding system, as described by Nirenberg & Leder (1964), is shown in figure 24. There was clearly a definite response, but the extent of this response was not the same for both species. The superior response by the ethanol species could have resulted from the lower specific activity of the salt species and the accompanying competition of deacylated tRNA. It could also have resulted from an inherent affinity of the ethanol species for the E.coli ribosomes or as stated earlier it may have reflected an effect of the low salt conditions.

The response towards poly (GU; 1:1) was also investigated and is shown in figure 25. There was again a significant response by both species to this template, and it was similar to that described for ApUpG in that the ethanol species was superior.

The artificiality of the binding assays and the need for carefully optimising conditions precluded a quantitative comparison of the behaviour of the species towards the two templates. In this sense the results of figures 24 and 25 were only qualitatively of value in that they clearly indicated a response by both species to both templates, the ethanol species being superior in both cases. It was therefore pertinent to determine a functional significance related to the recognition of ApUpG and poly (GU). This aspect was investigated in terms of the ability of poly (GU) to stimulate incorporation of methionine into polypeptide from both met-tRNAs and the ability of both met-tRNAs to form met-puromycin when bound to the ribosomes in the presence of ApUpG.

Figure 24

Ribosomal binding of H.cutirubrum met-tRNA species in response to ApUpG at varying Mg^{2+} in an E.coli system

E.coli ribosomal binding system as shown in table 7 (page 28).

Incubation at 26°C for 20 minutes.

Ribosomes : 2.68 A_{260} units.

Template : 0.11 A_{260} units of ApUpG (approx. 3 nmole).

Salt species : 0.23 A_{260} units, 1100 cpm., approx. 5.9 pmole.

Ethanol species : 0.06 A_{260} units, 1040 cpm., approx. 5.8 pmole.

Results :

	pmoles bound			
	SALT		ETHANOL	
mM Mg^{2+}	-ApUpG	+ApUpG	-ApUpG	+ApUpG
2	0	0	0.34	0.24
14	0.06	0.54	0.52	1.90
22	0.12	0.92	0.70	2.45
30	0.15	0.98	0.80	2.66
38	0.17	1.07	0.87	2.72

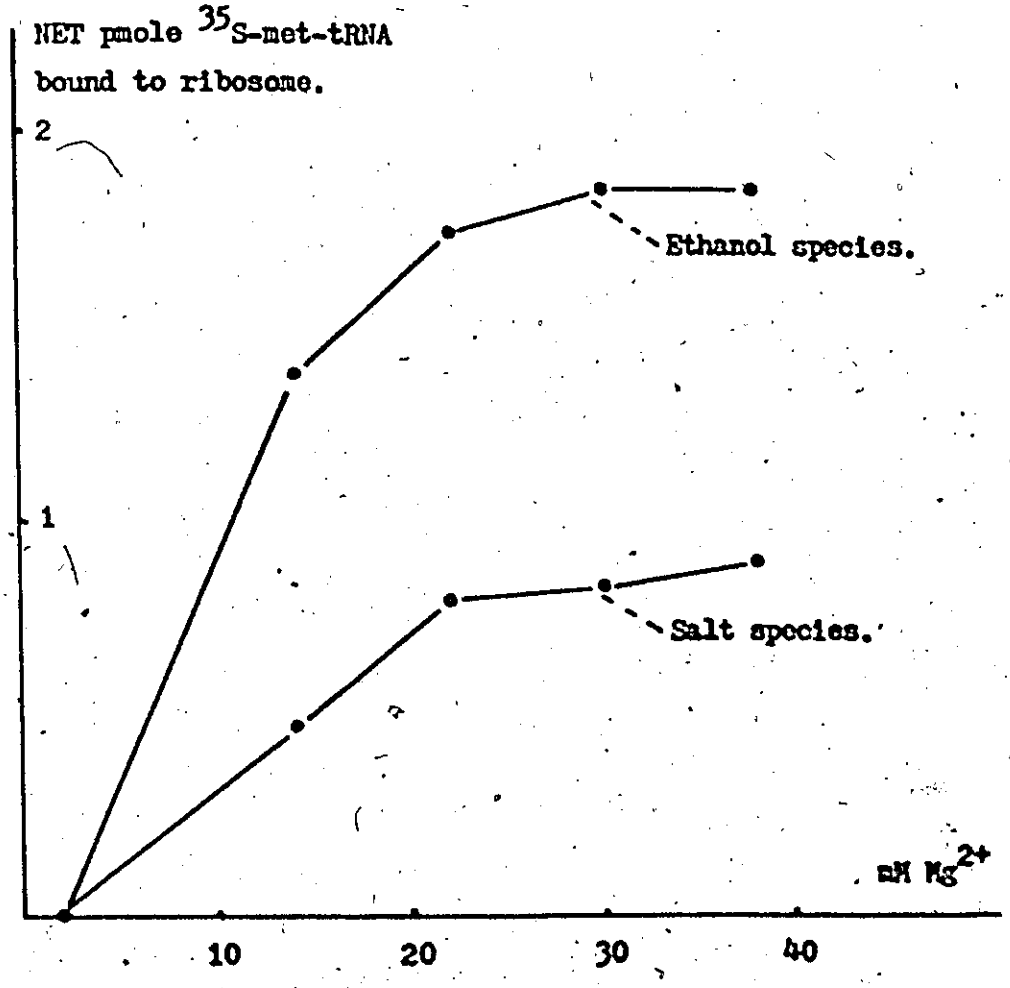

NET pmole ^{35}S-met-tRNA
bound to ribosome.
2
1
Ethanol species.
Salt species.
mM Mg^{2+}
10
20
30
40

Figure 25

Ribosomal binding of H.cutirubrum met-tRNA species in response to poly (GU) in an E.coli system

E.coli ribosomal binding system as shown in table 7 (page 28).

Incubation at 25°C for 20 minutes.

Ribosomes : 2.68 A_{260} units.

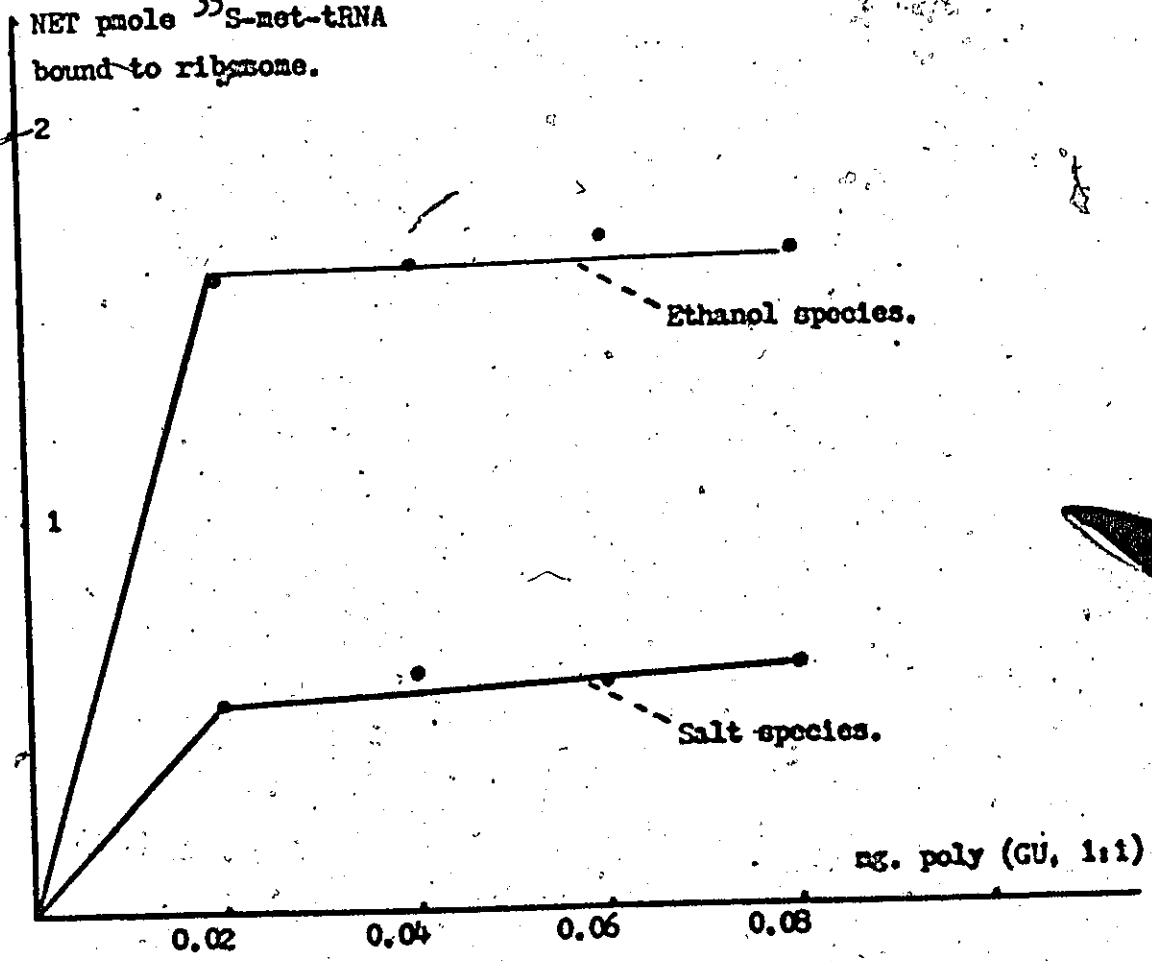
Mg^{2+} : 22mM

Salt species : 0.46 A_{260} units, 2200 cpm., approx. 11.8 pmole.

Ethanol species : 0.12 A_{260} units, 2080 cpm., approx. 11.6 pmole.

Results :

mg. poly (GU, 1:1)	pmole bound SALT	ETHANOL
0	0.06	1.08
0.02	0.58	2.70
0.04	0.66	2.73
0.06	0.63	2.80
0.08	0.68	2.76


NET pmole 35S-met-tRNA
bound to ribosome.
2
1
Ethanol species.
Salt species.
ng. poly (GU, 1:1)
0.02
0.04
0.06
0.08

5 In vitro initiation studies

11 Poly (GU) directed polypeptide synthesis

Clark & Marcker (1966) showed that in an E.coli polypeptide synthesising system met-$tRNA_f$ responded readily to a random poly (GU) template whilst met-$tRNA_m$ responded very poorly. Subsequent analysis of the polypeptide product indicated that met-$tRNA_f$ was positioning methionine N-terminally. A similar assay in an E.coli system was attempted using the H.cutirubrum met-tRNAm and is shown in figure 26. This assay used E.coli ribosomes and transferases but halophilic aminoacyl-tRNA. The result clearly demonstrated a response only by the ethanol species of ^{35}S-met-tRNA, which tentatively suggested an initiating capability for this species in E.coli. The result in figure 26 also indicated the capacity of H.cutirubrum tRNA to function in a low salt environment and thus provided further support for the conclusion that halophilic tRNA has not been grossly modified to function in high salt (White & Bayley, 1972b).

Attempts were made to utilise the met-tRNAs in an homologous H.cutirubrum synthesising system but they proved unsuccessful, as indicated in figure 27. No response by either of the species towards the poly (GU) template was observed, but this could not be ascribed as a negative response since Bayley & Griffiths (1968b) had clearly shown that methionine did respond to a random poly (GU) template. It appeared more likely that the lack of response was a consequence of two main factors, the limited efficiency of the H.cutirubrum incorporating system (White & Bayley, 1972a), and the rapid deacylation of aa-tRNA due to synthetase contamination of the transferase preparation (White & Bayley, 1972b). The presence of tRNA in the ribosomal preparation (Table 10, page 51) was

Figure 26

Poly (GU) directed polypeptide synthesis in an E.coli system

The reaction mixture was adapted from that of Clark & Marcker (1966). Each incubation contained the following components :- (0.100ml.)

30mM KCl, 10mM MgAc, 40mM Tris-HCl, pH 7.8, 2mM ATP, 6mM PEP, 0.9mM GTP, 0.05mg. pyruvate kinase and 20 ^{12}C-amino acids (8×10^{-5}M).

Ribosomes : 2.68 A_{260} units.

Transferase preparation (page 19) : 0.05mg. of protein.

Poly (GU, 1:1) : 0.025mg.

H.cutirubrum aa-tRNA : 0.5 A_{260} units of 19 ^{12}C-aa-tRNAs (- methionine).

Salt species : 3.2 A_{260} units, approx. 93 pmoles of ^{35}S-met-tRNA.

Ethanol species : 0.9 A_{260} units, approx. 87 pmoles of ^{35}S-met-tRNA.

Specific activity of ^{35}S-methionine was approximately 640 cpm./pmole.

Incubation at 30^{0}C.

Reaction tubes were treated exactly as for the H.cutirubrum polypeptide synthesising system (cf. page 20).

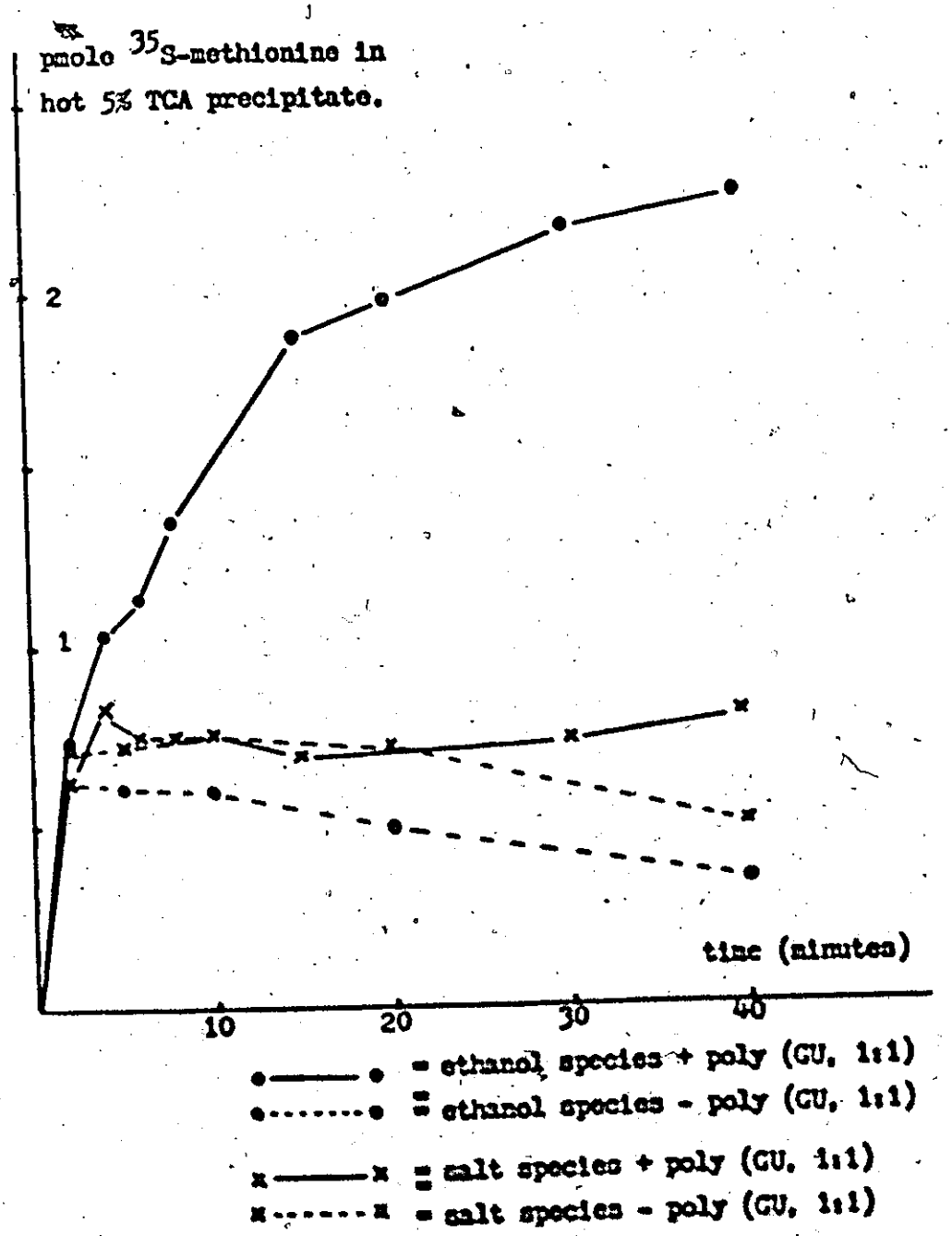

pmole 35S-methionine in
hot 5% TCA precipitate.
2
1
time (minutes)
10
20
30
40
● ——— ● = ethanol species + poly (GU, 1:1)
● ..... ● = ethanol species - poly (GU, 1:1)
x ——— x = salt species + poly (GU, 1:1)
x ..... x = salt species - poly (GU, 1:1)

Figure 27

Poly (GU) directed polypeptide synthesis in H.cutirubrum

The normal H.cutirubrum synthesising system was used (table 3, page 21).

The final conditions were as follows :-

3.8M KCl, 1M NaCl, 0.4M NH_4Cl, 40mM Mg^{2+}, 30mM Tris-HCl, pH 8.05,

1.5mM ATP, 6mM PEP and 0.9mM GTP.

Ribosomes : 2.88 A_{260} units.

Peak 1 (0.1 A_{260} units), from a G-75 Sephadex column of a 100% $(NH_4)_2SO_4$ precipitate of an S-150 was used as the source of transferases.

Poly (GU, 1:1) : 0.05mg.

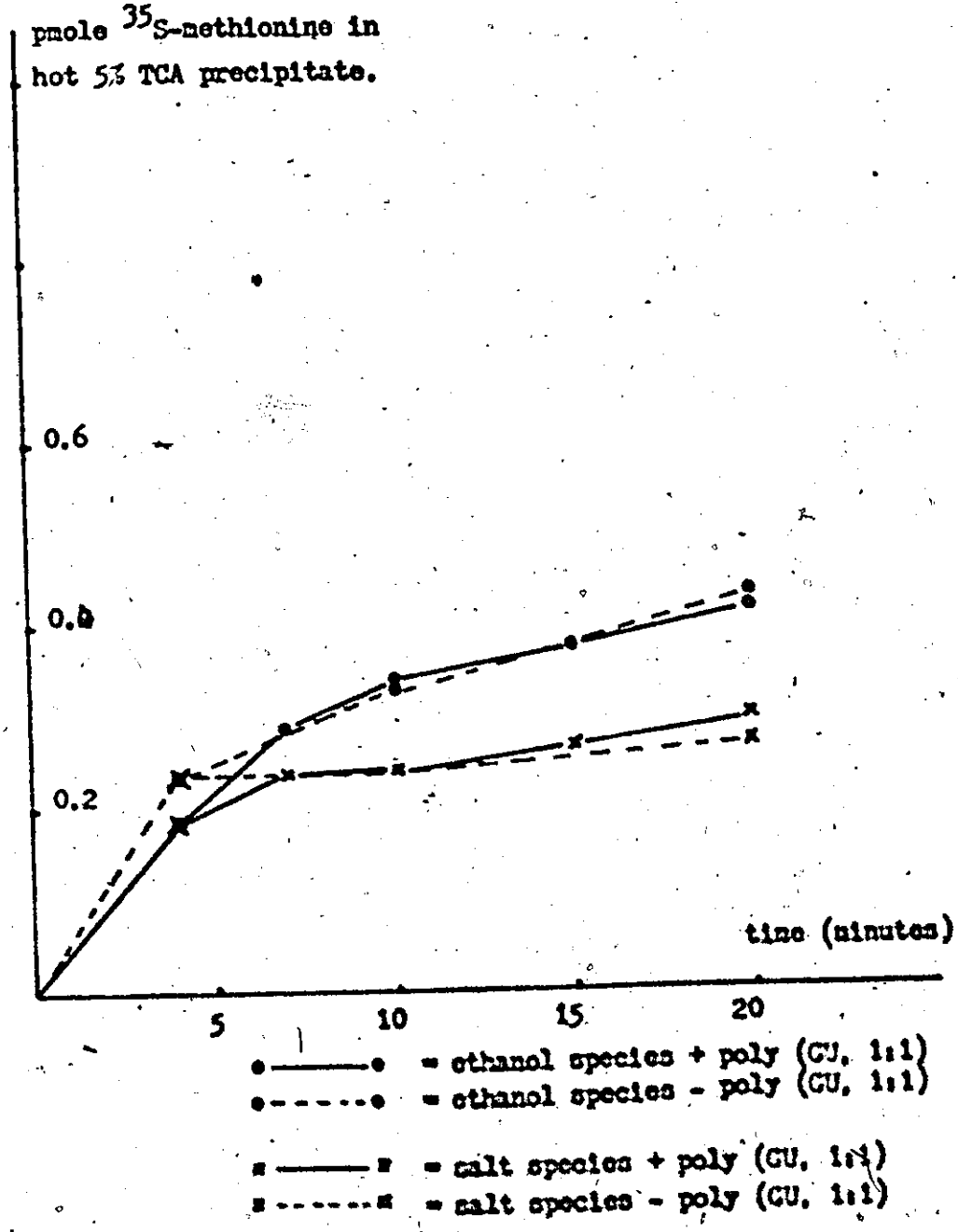
19 ^{12}C-aa-tRNAs (- methionine) : 0.25 A_{260} units.

Salt species : 1.60 A_{260} units, approx. 46 pmoles of ^{35}S-met-tRNA.

Ethanol species : 0.45 A_{260} units, approx. 43 pmoles of ^{35}S-met-tRNA.

Specific activity of ^{35}S-methionine was approximately 640 cpm./pmole.

Incubation at 37°C.


pmole ^{35}S-methionine in hot 5% TCA precipitate.
0.6
0.4
0.2
time (minutes)
5
10
15
20
• ——— • = ethanol species + poly (CU, 1:1)
• - - - - • = ethanol species - poly (CU, 1:1)
× ——— × = salt species + poly (CU, 1:1)
× - - - - × = salt species - poly (CU, 1:1)

an additional handicap to this assay system since it indicated that the addition of tRNA to the reaction mixture could not be selectively controlled. Attempts at utilising the met-tRNAs in an homologous incorporating system were not pursued and it was felt that such utilisation would in the future necessitate a much more refined in vitro polypeptide synthesising system.

An interesting observation concerning poly (GU) directed polypeptide synthesis was made during the course of the above studies and this related to its ability to direct synthesis at low Mg^{2+} concentrations. The result shown in figure 28 indicated that the optimum Mg^{2+} concentration for poly (GU) was approximately 14mM Mg^{2+}, a value which differed from the 20 - 40mM Mg^{2+} optimum determined for both endogenous and exogenous mRNA (Bayley & Griffiths, 1968a,b) and which was close to the 10mM Mg^{2+} required for the reversible dissociation of the bulk of 70S ribosomes (Rauser & Bayley, 1968). This suggested that polypeptide synthesis was possible at low Mg^{2+} as a consequence of poly (GU) possessing the initiating codon GUG. However, this proposal was not supported by later results which indicated, that the absence of methionine had no effect on the activity of poly (GU), the addition of the separated $tRNA^{met}$ species did not stimulate methionine incorporation and, as shown in table 19, poly U was also active as a template at 4mM Mg^{2+}, although to a lesser extent than poly (GU, 1:1). The reason for the increased activity of poly (GU) at low Mg^{2+} could therefore not be directly attributed to the possession of an initiating GUG codon and whether this is an initiating codon in the halophilic system remains to be determined.

Figure 28

^{14}C-phe. incorporation into polypeptide in response to poly U and poly (GU) at varying Mg^{2+} concentrations

Incubation at 37°C for 40 minutes in normal H.cutirubrum polypeptide synthesising system (table 3, page 21).

Ribosomes : unwashed ribosomes were diluted in 3M KCl, 0.1M Tris-HCl, pH 7.6, and 1mM MgAc, pelleted by centrifugation at 150,000g. for 2.5 hours and resuspended in the above buffer. 0.27mg. of ribosomes were used per incubation mixture.

S-150 (0.25mg. of protein) was used as source of synthetases and transferases.

H.cutirubrum unfractionated tRNA : 1 A_{260} unit per assay.

Poly U : 0.25mg.

Poly (GU, 1:1) : 0.6mg.

The specific activity of ^{14}C-phenylalanine was not known and therefore the results opposite are shown as a % incorporation, with the highest value (poly GU at 14mM Mg^{2+}) taken as 100%.

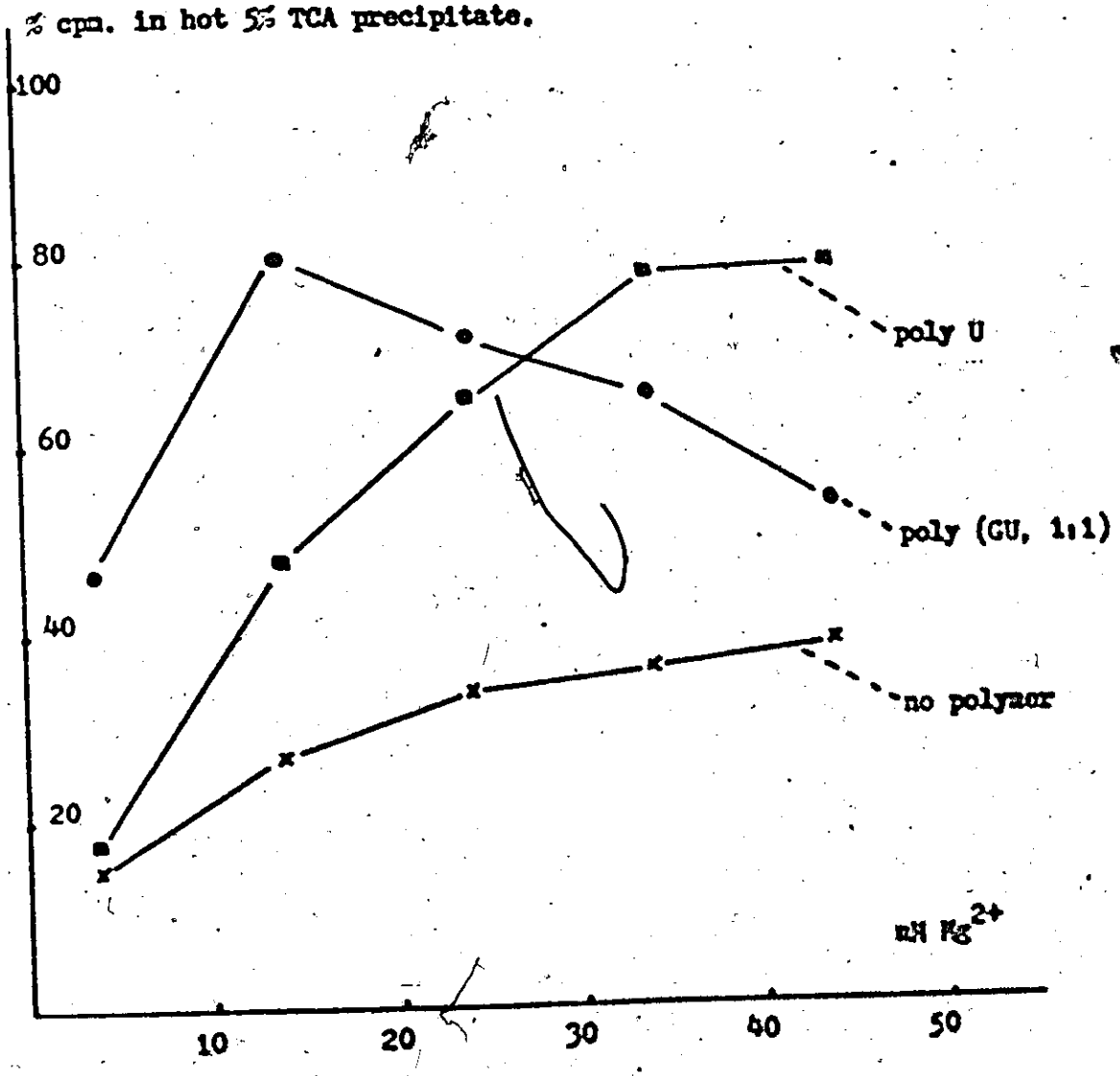

% cpm. in hot 5% TCA precipitate.
100
80
60
40
20
poly U
poly (GU, 1:1)
no polymer
mM Mg^{2+}
10
20
30
40
50

Table 19

^{14}C-phe. incorporation into polypeptide in response to poly U and poly (CU) at 4mM Mg^{2+}

reaction mixture	cpm. per mg. ribosome in hot 5% TCA-precipitate	% incorporation
complete * + poly (CU)	33400	100
without poly (CU)	4350	13
without added tRNA	20700	62
with puromycin	550	1.6
complete ** + poly U	13500	40
without poly U	3400	10

*,** separate experiments

*** incorporation with poly (CU) arbitrarily set as 100%.

Incubation at 37°C for 40 min. in normal *M.ortiriubrum* synthesising system (table 3, page 21). Pre-incubated ribosomes (page 15) were stored in 3% NH4Cl, 0.1M Tris-HCl, pH 7.6, and 1mM Mg^{2+}.

Poly U, 0.05mg. ; poly (CU, 1:1), 0.05mg. ; 1 A_{260} unit of *M.ortiriubrum* unfractionated tRNA ; S-150 (0.25mg. of protein) used as source of synthetases and transferases ; puromycin, 0.025mg.

Unknown sp. act. ^{14}C-phe. (estimate : 66 mc/mmole).

5 In vitro initiation studies

111 Methionyl-puromycin formation

Experiments in other organisms dealing with initiation made extensive use of puromycin. This antibiotic is thought to bind close to the aminoacyl-tRNA binding site, the "A" site, and then react via the peptidyl transferase with the polypeptidyl-tRNA in the polypeptidyl-tRNA binding site, the "P" site. In the case of initiation it is believed that sensitivity towards puromycin is a consequence of an initiator tRNA being in the P site and that non-initiating aminoacyl-tRNAs are comparatively insensitive to puromycin since they would normally be present in the A site (Brotscher & Marcker, 1966). This sensitivity to puromycin was specifically adapted for study of the initial peptide bond by Leder & Buratyn (1966). Their approach in E.coli involved a two step reaction: binding of aminoacyl-tRNA in response to its specific triplet, followed by the addition of puromycin, whose effect was monitored by release of aminoacyl-puromycin which was selectively extracted into ethyl acetate.

In light of the appreciable binding of H.cutirubrum met-tRNAs observed with E.coli ribosomes, the puromycin reaction was initially attempted in the E.coli system. As shown in table 20 neither of the H.cutirubrum species reacted with puromycin. The unfractionated, non-formylated E.coli met-tRNAs did respond to puromycin and it was felt that the magnitude of the response was sufficient to allow for the detection of met-puromycin if either of the H.cutirubrum species had reacted.

The H.cutirubrum ribosomal binding system suffered from two drawbacks which prevented the use of the two step reaction system as described for E.coli. These were the poor response to AUG and the

Table 20

Formation of met-puromycin on E.coli ribosomes

tRNA	pmole met-tRNA bound to ribosome	pmole in ethyl acetate	
		+puromycin	-puromycin
E.coli met-tRNAs	53	1.80	0.50
salt species	16	0.45	0.45
ethanol species	30	0.35	0.35

Two step reaction :

1 Normal binding reaction (table 7, page 28), 20 min. at 24°C. reaction tube cooled to 0°C, addition of puromycin (final conc^{n}. 6mM)

2 Puromycin reaction, incubation at 37°C for 30 minutes.

ethyl acetate extraction at pH 8.1 (page 24)

reaction conditions : 24mM Mg^{2+}

E.coli met-tRNAs : 0.250ml.

washed ribosomes (page 19), 26.8 A_{260} units; ApUpG, 26.25 nmoles.

unfractionated ^{14}C-met-tRNA, 7.1 A_{260} units, 90,000 cpm., 375 pmoles.

salt species : 0.500ml.

washed ribosomes, 53.6 A_{260} units; ApUpG, 52.5 nmoles.

^{35}S-met-tRNA, 7.6 A_{260} units, 35,400 cpm., 196 pmoles.

ethanol species : 0.300ml.

washed ribosomes, 32.1 A_{260} units; ApUpG, 31.5 nmoles.

^{35}S-met-tRNA, 0.72 A_{260} units, 12,500 cpm., 69.5 pmoles.

considerable deacylation, approximately 85%, which occurred at 37°C over the 40 min. incubation period. However an assay (page 24) similar to that described by Bretscher & Marcker (1966) was attempted and is described in table 21. In this assay, puromycin was present throughout the 40 min. incubation and this had the advantage of augmenting the effective response to ApUpG.

The result indicated that puromycin increased the amount of cpm. extracted into ethyl acetate by the ratios x2, x4 and x6 for the salt species, ethanol species and E.coli species respectively. The ratios were considered to represent met-puromycin formation and using this criterion the values for the ethanol species were consistently higher than those for the salt species by a factor of approximately 2. It was also worthy of note from this experiment that the unfractionated E.coli met-tRNA was capable of forming met-puromycin on the H.cutirubrum ribosomes and even to a greater extent than on E.coli ribosomes (cf. table 20). Methionyl-puromycin formation by the H.cutirubrum met-tRNAs was further analysed in terms of the components necessary, table 22, and the time course, figure 29.

This puromycin reaction was designed as a direct assay for polypeptide chain initiators and the results described for the H.cutirubrum system indicated that the ethanol species was better than the salt species in its capacity to react with puromycin. Positive identification of the ethanol species as an initiator would require a much more thorough analysis of this reaction and the first stage in this process would be the development of an H.cutirubrum ribosomal binding system capable of allowing a significant response to the trinucleotide ApUpG.

Table 21

Formation of met-puromycin on H.cutirubrum ribosomes

tRNA	pmole met-tRNA bound to ribosomes		pmole in ethyl acetate	
	+puro	-puro	+puro	-puro
salt species	1.89	3.70	2.11	0.95
ethanol species	1.83	2.55	1.31	0.33
E.coli met-tRNAs	4.90	6.20	6.50	1.10

Incubation at 37°C for 40 minutes in normal H.cutirubrum binding system (table 5, page 25) in presence of puromycin (1.35mM). Ethyl acetate extraction at pH 8.1 (page 24).

reaction conditions : 8mM Mg^{2+}.

Salt species : 0.500ml.

pre-incubated ribosomes (page 15). 23 A_{260} units; ApUpG, 21 nmoles.

^{35}S-met-tRNA, 7.6 A_{260} units, 35,400 cpm., 196 pmoles.

Ethanol species : 0.375ml.

pre-incubated ribosomes, 17.3 A_{260} units; ApUpG, 15.7 nmoles.

^{35}S-met-tRNA, 0.70 A_{260} units, 12,800 cpm., 71 pmoles.

E.coli met-tRNAs : 0.250ml.

pre-incubated ribosomes, 11.5 A_{260} units; ApUpG, 10.5 nmoles.

unfractionated ^{14}C-met-tRNA, 7.1 A_{260} units, 90,000 cpm., 375 pmoles.

Table 22

Conditions for met-puromycin formation on H.cutirubrum ribosomes

reaction conditions	cpm. extracted into ethyl acetate	
	salt species	ethanol species
complete	180	190
- ApUpG	155	180
- ribosomes	145	110
- puromycin	125	60

Incubation at 37°C for 45 minutes in normal H.cutirubrum binding system (table 5, page 25) in presence of puromycin (1.35mM). Ethyl acetate extraction at pH 8.1 (page 24).

reaction mixtures : 88mM Mg^{2+}, 0.125ml.
pre-incubated ribosomes (page 15). 5.6 A_{260} units; ApUpG, 0.75 nmoles.
salt ^{35}S-met-tRNA, 0.96 A_{260} units, 15,270 cpm., 24 pmoles.
ethanol ^{35}S-met-tRNA, 0.27 A_{260} units, 14,250 cpm., 22.3 pmoles.

Deacylation of met-tRNA in reaction mixture without ribosomes, ApUpG, and puromycin was 91% for the salt species and 92% for the ethanol species.

Figure 29

Formation of met-puromycin on H.cutirubrum ribosomes

The procedure was followed according to page 24 and the reaction conditions (table 5, page 25) involved :-

Ribosomes : 5 A_{260} units.

ApUpA : 1.5 nmoles.

Puromycin : final concentration of 2mM.

Mg^{2+} : 8mM.

Salt species : 1.6 A_{260} units, approx. 40 pmoles of ^{35}S-met-tRNA.
approx. 30,500 Aquasol soluble cpm.

Ethanol species : 0.45 A_{260} units, approx. 37 pmoles of ^{35}S-met-tRNA.
approx. 29,200 Aquasol soluble cpm.

Incubation at 37°C for the times indicated.

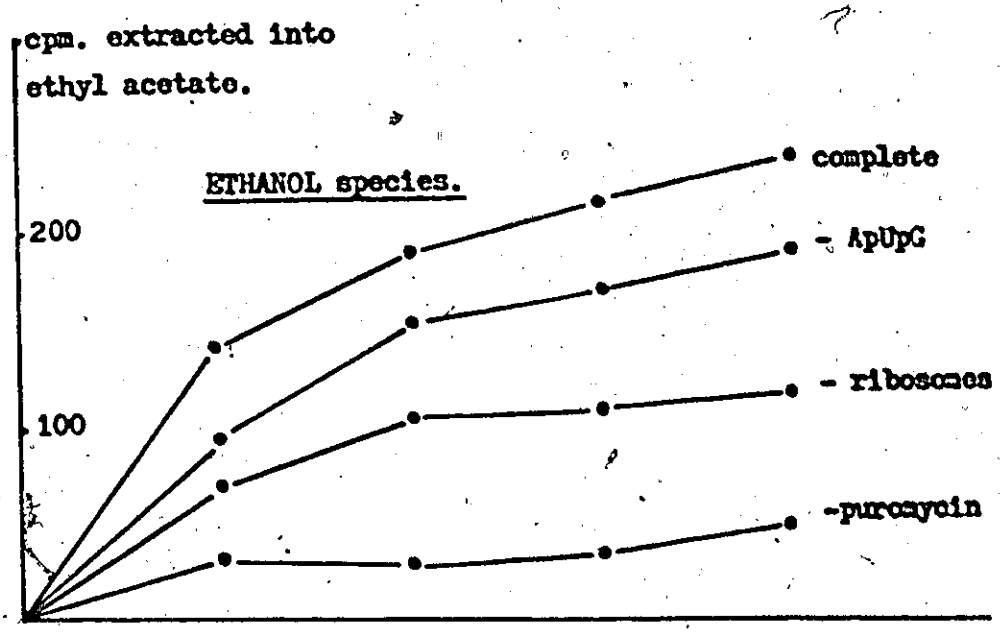
cpm. extracted into ethyl acetate at time zero :-

Salt species : approx. 10

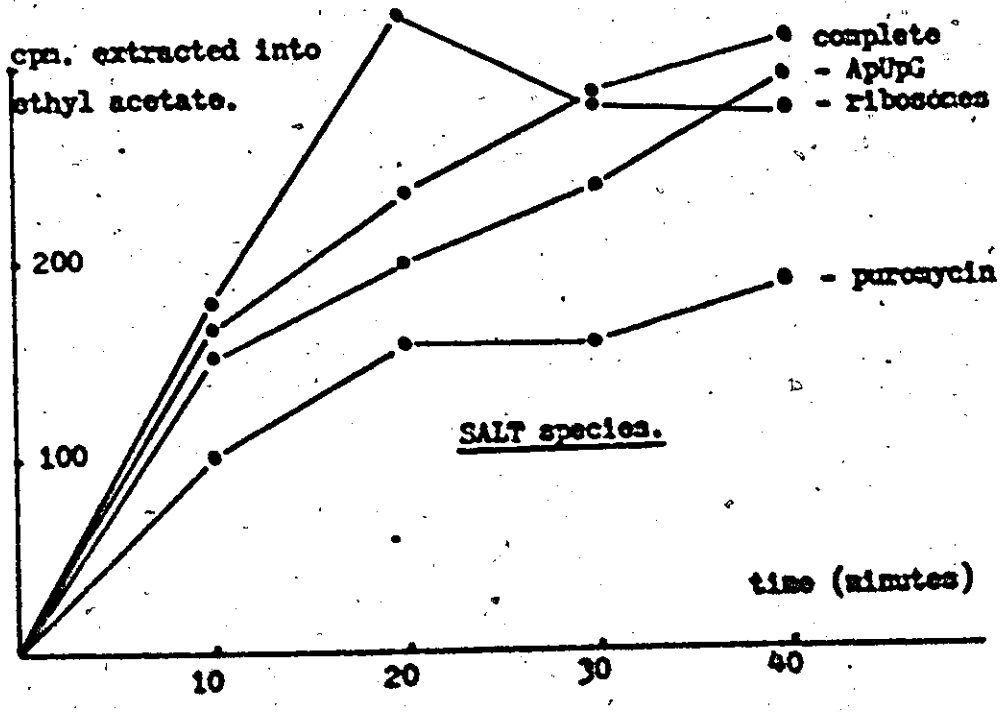
Ethanol species : approx. 20

These values have been subtracted, providing the results shown opposite.

a.


cpm. extracted into
ethyl acetate.
ETHANOL species.
200
100
complete
- ApUpG
- ribosomes
-puromycin

b.


cpm. extracted into
ethyl acetate.
200
100
SALT species.
time (minutes)
10
20
30
40
complete
- ApUpG
- ribosomes
- puromycin

V Discussion

The aim of this thesis was to investigate translational initiation in H.cutirubrum but the bulk of the work was devoted to the isolation of the two $tRNA^{met}$ species. The major difficulties encountered were the poor yield and purity of the transfer RNA and the lack of chromatographic resolution on BD-cellulose; both of these problems are described in section 1. The problem of chromatographic resolution was principally a consequence of the poor quality of the original BD-cellulose but was probably aggravated by the large amounts of RNA and DNA, contaminants of the tRNA, interfering with the binding of methionyl-tRNA. The poor tRNA product was considered a result of the losses occurring during the isolation procedure and the damage which likely ensued from this process. The approaches which led eventually to the isolation of the $tRNA^{met}$ species (fig. 8a, page 61) are described in section 2, but the key factors were the improvement in the later commercial preparations of BD-cellulose and the development of a new preparative procedure for tRNA.

The new tRNA was approximately 65% pure, as judged by the acceptance of amino acids (table 12, page 57) whilst the old procedure had resulted in a product composed of 9% tRNA, 65% RNA with no acceptance activity, 20% DNA and 6% protein (cf. page 50). It was therefore felt that the new product was a much more reliable representation of native tRNA and was free of the artifacts present in the old tRNA preparation. The chromatographic resolution of the $tRNA^{met}$ species, using the new tRNA product, was thoroughly investigated to determine the proportion of the two species and the basis for their separation (section 31).

With the new tRNA, the ethanol $tRNA^{met}$ was the major species whilst previous reports (B.N.White, 1970) had indicated that the salt species was in excess. This discrepancy was attributed to the different tRNA preparations and the new proportion of species proved to be a consistent feature (e.g. with variations in charging, chromatographic conditions and age of H.cutirubrum cells). H.cutirubrum was therefore considered to possess two $tRNA^{met}$ species, approximately 35% being the salt species ($tRNA_1^{met}$) and 65% the ethanol species ($tRNA_2^{met}$). The behaviour of the $tRNA^{met}$ species on Deae-Sephadex, RPC-5 and BD-cellulose (figs. 8 & 9, pages 61 & 65) provided information on the structural features which controlled their chromatographic separation. The results suggested that $tRNA_1^{met}$ possessed a more rigid and compact structure than $tRNA_2^{met}$, that $tRNA_2^{met}$ was more hydrophobic, either as a consequence of its tertiary structure or its possession of modified bases, and that hydrophobicity was the critical factor in their chromatographic separation.

The work with $tRNA^{met}$, as discussed above, indicated that differences arose between the old and new tRNA preparations. This in turn raised the broader question of whether the development of a new isolation procedure for tRNA would demonstrate properties for halophilic tRNA different from those in previous reports (Griffiths & Bayley, 1969; White & Bayley, 1972a, b, c). A detailed comparison of the old and new tRNA was not attempted but in those areas which overlapped previous work the behaviour of the tRNA was similar and the differences reflected the increased purity of the new tRNA. The ability of the halophilic aa-tRNA to function in an E.coli synthesising system (fig. 26, page 118) lent further support to the basic conclusion of White & Bayley (1972b) that halophilic tRNA has not been grossly modified to function in high salt.

It is relevant at this stage, before turning to those results which relate to initiation, to briefly summarise those points which suggest an initiator function for $tRNA_2^{met}$. Methionine has been shown to be an initiating amino acid in a wide variety of organisms (Bretscher, 1971), results with H.cutirubrum have so far been in total agreement with the concept of a universal genetic code (White & Bayley, 1972a), methionine responded to poly (GU) in an H.cutirubrum polypeptide synthesising system (Griffiths & Bayley, 1968b), and finally, H.cutirubrum $tRNA_2^{met}$ was formylatable in an E.coli system even though no f-met-tRNA nor a formylating system was detected in H.cutirubrum (White & Bayley, 1972c).

A direct in vivo attempt at implicating methionine in an initiating role was made using ^{35}S-methionine and puromycin as described in section 4. The experiments were inconclusive but they did show that two radioactive compounds, as yet unidentified, resulted from the addition of puromycin and that one of these compounds had an electrophoretic mobility similar to that of methionyl-puromycin. Several reasons could have explained the poor quantitative response. The rationale behind these experiments was that puromycin would be present in sufficient amounts to cause both the release of polypeptide chains and chain termination immediately after subsequent reinitiation (Bachmayer & Kreil, 1968); the formation of an initiator amino acid - puromycin complex would therefore be subject to certain constraints. Amongst these would be whether there was sufficient puromycin present within the cell, whether there was an active amino-peptidase present, and finally whether reinitiation could have simply occurred at the next codon and not required the normal initiation complex (Friedman et al, 1968; Williamson & Schweet, 1965). The experiments therefore neither

supported nor disproved the possibility of methionine being the initiator amino acid.

The remainder of the information concerning initiator activity was indirect and it involved those features of the halophilic $tRNA^{met}$ species which compared with similar properties of propagator and initiator $tRNA^{met}$ species from other organisms.

The results provided in section 3 ii clearly indicated that $tRNA_1^{met}$ was not recognised by an E.coli synthetase, while $tRNA_2^{met}$ was recognised and its elution pattern was not dramatically altered after hererologous acylation. These results supported previous findings which showed that $tRNA^{met}$ was both acylatable and formylatable in an E.coli system and that met-$tRNA_2^{met}$ was the formylatable species, (B.N.White, 1970). They did not, however, explain the drastic alteration in the BD-cellulose elution profile, after heterologous acylation, where with tRNA prepared by the old method two met-tRNA peaks were not resolved under elution conditions suitable for the resolution of homologously charged species (B.N.White, 1970). Possible explanations for the latter behaviour were that damaged tRNA was present in the old tRNA, and/or modifying enzymes, in the different E.coli S-150 preparations, introduced conformational changes in the $tRNA^{met}$ structure.

It seems clear that $tRNA_2^{met}$ is charged by an E.coli enzyme but the problem arises whether gross modification of this tRNA is necessary before formylation. If this is necessary, then the fact that $tRNA_2^{met}$ is formylatable in an E.coli system might be totally artificial and may have no bearing on initiation. It still remains to be determined whether $tRNA_2^{met}$ can be formylated by the E.coli transformylase without gross modification of its native conformation. A positive aspect of heterologous E.coli

acylation lies in the fact that $tRNA_2^{met}$ was charged and $tRNA_1^{met}$ was not charged. This compares favourably with the findings of Ghosh et al (1971) and Bhaduri et al (1970) where with wheat embryo and rabbit reticulocytes, respectively, the initiator $tRNA^{met}$ was acylated whilst the propagator $tRNA^{met}$ was not recognised.

RNAse-T_1 digestion of H.cutirubrum met-tRNA resulted in two different 3' terminal fragments (fig. 16, page 87); upon electrophoresis at pH 3.5 the $tRNA_1^{met}$ fragment moved to the cathode whilst the $tRNA_2^{met}$ fragment moved towards the anode. Their electrophoretic mobilities were remarkably similar to those reported for the respective propagator and initiator $tRNA^{met}$ species of mouse liver, yeast, mouse ascites tumour cells (Smith & Marcker, 1970), and E.coli (Marcker, 1965). Although no particular function has been suggested for the 3' terminal fragment it is interesting that all of the latter four initiator tRNAs (Smith & Marcker, 1970) and H.cutirubrum $tRNA_2^{met}$ (White & Bayley, 1972c) can be formylated by an E.coli transformylase and transformylase recognition is dependent upon the structure of tRNA (Giege, Ebel & Clark, 1973). It is further interesting to note that the f-met-T_1 digest fragment of E.coli f-met-$tRNA_f^{met}$ is capable of forming f-met-puromycin on E.coli 50S ribosomes (Monro & Marcker, 1967).

If indeed $tRNA_2^{met}$ is an initiator tRNA, then the order of elution on DD-cellulose is reversed from that of yeast, E.coli, mouse liver, mouse ascites tumour cells, rabbit reticulocytes, H.laidlawii, S.faecalis R (Samuel & Rabinowitz, 1972a) and wheat embryo (Leiss & Keller, 1970). Any attempt to assign significance to this reversal is subject to the proviso that the chromatographic conformation of $tRNA_2^{met}$ is not necessarily

the native conformation. However, two interesting aspects of this apparent reversal are that, if there is a common structural feature which chromatographically distinguishes between initiator and propagator $tRNA^{met}$ species then this is not present in H.cutirubrum $tRNA^{met}$ species, and two cases have been reported where the elution order has been reversed by modification of met-tRNA. Heneo et al (1969) chromatographed the phenoxyacetyl derivatives of E.coli met-tRNA and Samuel & Rabinowitz (1972a) used formylated S.faecalis R met-tRNA and in both cases the initiator species was eluted after the propagator. A proposed explanation for this reversal was that blockage of the alpha amino group induced a conformational change within the met-tRNA. Dube et al (1969) suggested that bacterial initiation factors possess a specificity for an N-blocked amino group and the initiator $tRNA^{met}$ structure and therefore it is interesting to speculate that H.cutirubrum $tRNA_2^{met}$ possesses a critical conformation without the necessity of a blocked alpha amino group.

The best evidence regarding $tRNA_2^{met}$ initiator activity came from studies on the codon recognition (section 5i) and poly (GU) directed synthesis (section 5ii). However, these experiments were carried out in E.coli systems and must accordingly be considered as essentially artificial. $tRNA_2^{met}$ was shown to respond both to AUG and poly (GU) in ribosomal binding assays (figs. 24 & 25, pages 113 & 115) and also to poly (GU) in a polypeptide synthesising system (fig. 26, page 118). This suggested that AUG and GUG were codons for $tRNA_2^{met}$ and that $tRNA_2^{met}$ responded to a 5' terminal GUG. These two properties are indicative of an initiator tRNA (Bretscher, 1971). Unfortunately these results could not be repeated in the H.cutirubrum systems, but the reasons for this were not considered physiological, rather they were a consequence of the limited efficiency of the halophilic

in vitro systems. The formation of not-puromycin on halophilic ribosomes (table 21, page 128) however, tentatively suggested an initiator function for $tRNA_2^{met}$ in H.cutirubrum. The small extent of formation and the high background response of $tRNA_1^{met}$ prevented a more definitive conclusion but the response was consistent enough to indicate the value of this technique for future initiation studies.

Section 5 also illustrated the main problems encountered in attempting to assign functional roles to halophilic tRNA. The limited efficiency of H.cutirubrum in vitro assay systems is a major stumbling block and it is not known whether this is an inherent feature or whether it may be improved by further purification of the components. The latter would be a time consuming and difficult process as the components often require high salt concentrations for stability thus preventing the use of conventional purification techniques. Also the high salt conditions required for activity are often themselves reason for the poor activity. The inability of templates with high adenine content to function as messengers was due to their precipitation under the assay conditions (B.N.White, 1970), and aggregate formation may be the reason why triplet templates do not provide significant response in halophilic binding systems. The poor response in halophilic systems has led to the use of E.coli in vitro assays and the results obtained accordingly require a very thorough analysis before a conclusion can be drawn relative to the halophilic environment. This latter aspect is particularly relevant to halophilic $tRNA_1^{met}$ and $tRNA_2^{met}$; for although the E.coli system provided results supportive of an initiator role for $tRNA_2^{met}$ the possibility of non-physiological behaviour is still a critical factor.

A considerable body of circumstantial evidence now indicates that initiation in H.cutirubrum involves a non-formylated species of met-tRNA ($tRNA_2^{met}$). It is therefore interesting to consider why H.cutirubrum should be an exception to normal prokaryotic initiation. Forsyth & Kushner (1970) concluded, from a study of the nutritional requirements of moderate halophiles, that the greater nutritional requirements found at high salt concentration were due to the inhibition of enzymes responsible for the synthesis of amino acids and other growth factors. Thus it is conceivable that halophilic adaptation has been one of coping with adversity and that the increase in salinity was accompanied by loss of enzyme activity. The complex nutritional requirements of the extreme halophiles may reflect this loss of activity. The role of the formyl group in prokaryotic initiation appears to be the major factor for recognition by bacterial initiation factors (Brown et al, 1973) whilst eukaryotic systems accomplish the same process by virtue of a unique initiator $tRNA^{met}$ structure (Ghosh & Ghosh, 1972). Therefore during the evolution of halophiles either the initiator $tRNA^{met}$ structure was modified and this obviated the requirement for a formyl group, and hence the transformylase, or transformylase activity was inhibited and this in turn led to modification of the initiator $tRNA^{met}$. The latter possibility appears the more likely.

Support for the conclusion that $tRNA^{met}$ modification occurred after transformylase inhibition comes from observations with S.faecalis R (Samuel & Babinowitz, 1972b) and E.coli (Harvey, 1973) where transformylase activity is prevented in the former case by lack of folate in the growth medium and in the latter case by the addition of trimethoprim. In both cases protein synthesis appears to be initiated with a non-formylated initiator ($tRNA^{met}$) and a new chromatographically distinct species of

$tRNA_f^{met}$ was obtained with S.faecalis R.

The ability to isolate the H.cutirubrum $tRNA^{met}$ species on a routine basis and the techniques for purification of $tRNA_2^{met}$ have been demonstrated in this thesis. It is therefore possible for definitive conclusions concerning the initiator role of $tRNA_2^{met}$ to be obtained.

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VII APPENDIX

1 Analysis of preparative procedure for transfer RNA

G-100 Sephadex gel filtration of the old tRNA preparation (figures 7a,b, page 53) indicated that acceptance activity was present only in peak 2. Therefore G-100 Sephadex chromatography was used to monitor the loss of peak 2 material at various stages in the preparative procedure.

An analysis of the old preparative procedure is shown in figure 30. Figures 30 (abcd) were obtained from the same preparation whilst figure 30e was from a separate experiment. Losses of peak 2 material clearly occurred during the centrifugation step to pellet the ribosomes and during the pH 5 precipitation of synthetases.

An analysis of the new preparative procedure is shown in figure 31. This served not only to determine the effectiveness of the isopropanol fractionation but also it provided a G-100 Sephadex elution profile for the new tRNA product. Figure 31e may be compared directly with the elution profiles of the old tRNA (figs. 7a, b, page 53). The new tRNA was approximately 60% pure as judged by the acceptance of amino acids (table 12, page 57) and approximately 92% of the A_{260} units eluted as peak 2 on G-100 Sephadex.

Figure 30

G-100 Sephadex analysis of old tRNA (prop^{n}. a, page 17)

18 hour culture (24L., 5% inoculum) : yield 26g. wet weight of cells.

Profiles abcd follow the A_{260} units through the extraction procedure.

G-100 Sephadex columns : 2.5 x 40 cm., 4°C, 36 ml./hr., 2ml. fractions.

a. S-60 extract : total A_{260} units - 6500

G-100 Sephadex - 260 A_{260} units applied, solution D' used for elution.

Distribution - peak 1 - 80%, peak 2 - 10%, peak 3 - 10%.

b. S-150 extract : total A_{260} units - 1600

G-100 Sephadex - 60 A_{260} units applied, solution D' used for elution.

Distribution - peak 1 - 45%, peak 2 - 25%, peak 3 - 35%.

c. Ribosomes in high salt : total A_{260} units - 1200

G-100 Sephadex - 150 A_{260} units applied, solution D' used for elution.

Distribution - peak 1 - 98%, peak 2 - 2.5%, peak 3 - 0.5%.

d. Ribosomes in low salt : total A_{260} units - 1200

G-100 Sephadex - 125 A_{260} units applied, 10mM Ac, pH 4.5, used for elution.

Distribution - peak 1 - 95%, peak 2 - 4.5%, peak 3 - 0.5%.

The pH 5 synthetase preparation was from a separate procedure.

e. pH 5 synthetases

G-100 Sephadex - 40 A_{260} units applied, solution D' used for elution.

Distribution - peak 1 - 55%, peak 2 - 45%.

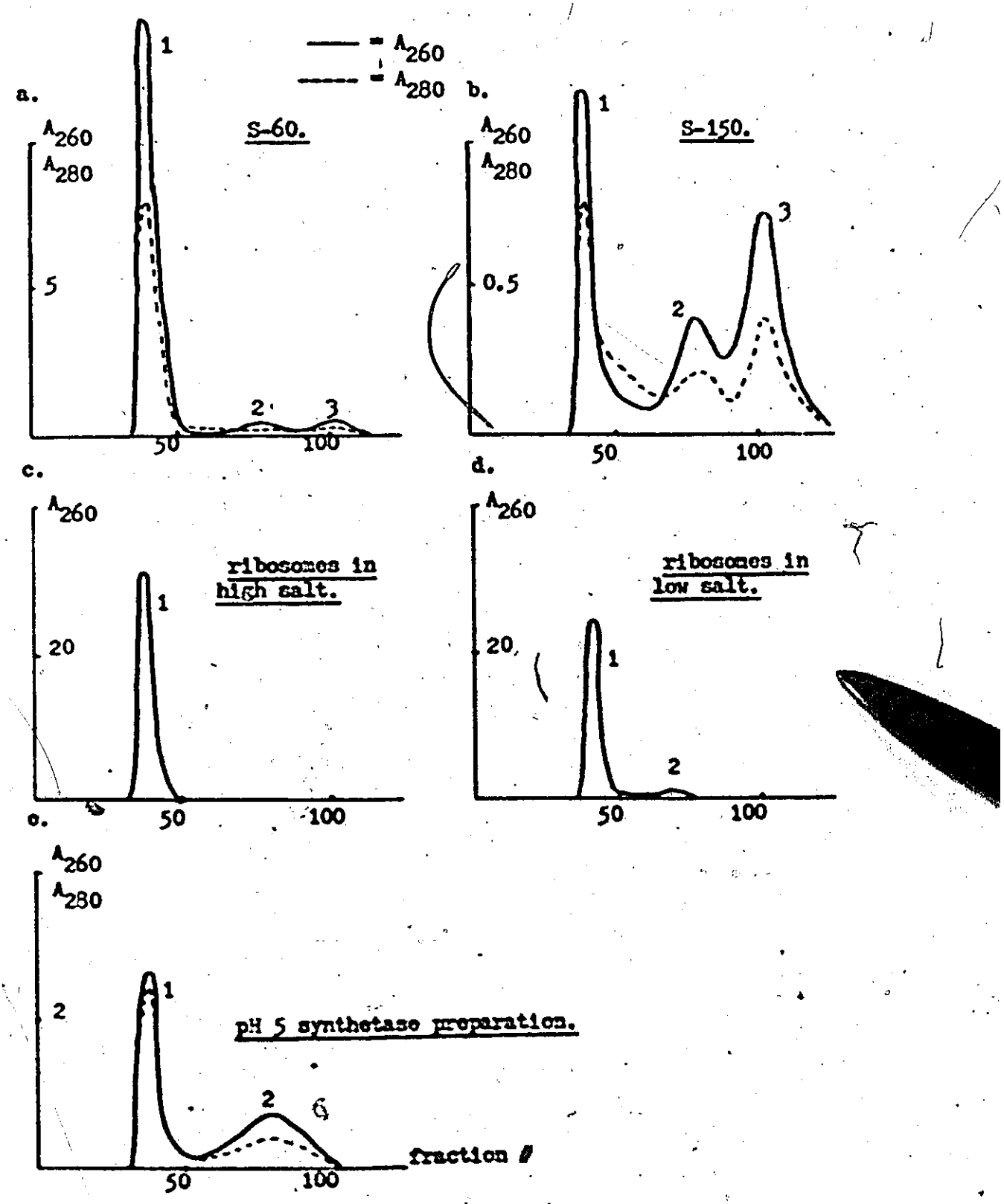

a.
S-60.
A260
A280
5
1
2
3
50
100
b.
S-150.
A260
A280
0.5
1
2
3
50
100
c.
ribosomes in high salt.
A260
20
1
50
100
d.
ribosomes in low salt.
A260
20
1
2
50
100
e.
A260
A280
2
1
2
50
100
pH 5 synthetase preparation.
fraction #

Figure 31

G-100 Sephadex analysis of new tRNA (prep^{n}. b, page 18)

43 hour culture (24L., 5% inoculum) : yield 76g. wet weight of cells.

G-100 Sephadex columns : 2.5 x 36 cm., 4°C, 36 ml./hr., 2ml. fractions, 10mM NAc, pH 4.5, used for elution.

a. Total nucleic acid : 44,000 A_{260} units (3g.)

Composition (by weight) 25 - 30% DNA. 65% RNA.

G-100 Sephadex - 48 A_{260} units applied.

Distribution - peak 1 - 90%, peak 2 - 10%.

An isopropanol fractionation separated tRNA from the high MW nucleic acid.

b. High MW nucleic acid : 40,000 A_{260} units (2.5g.)

Composition (by weight) 35% DNA 60% RNA.

G-100 Sephadex - 47 A_{260} units applied.

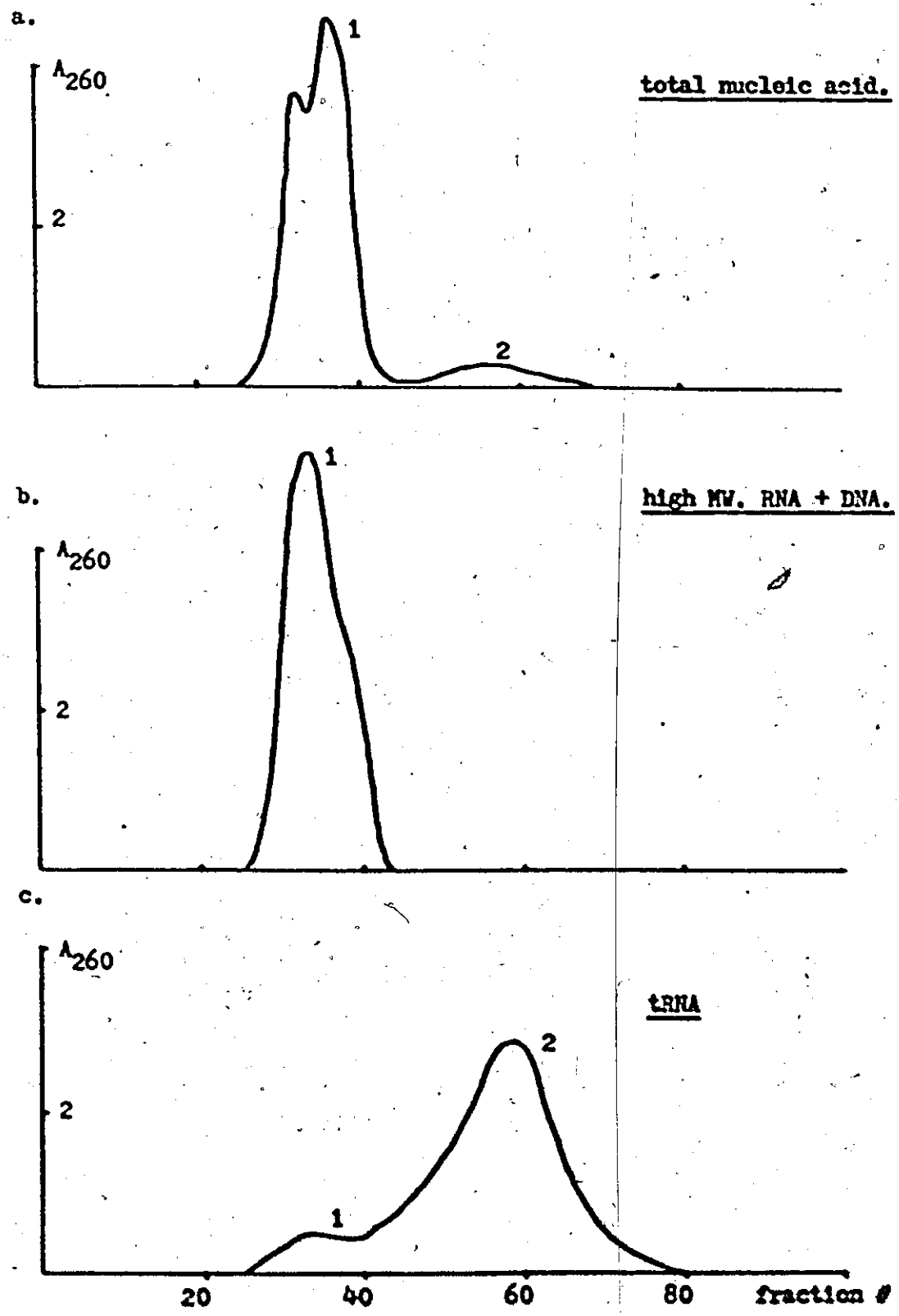
Distribution - peak 1 - 99%, peak 2 - 1%.

c. tRNA : 3,500 A_{260} units (170mg.)

Composition (by weight) 4% protein 90% RNA.

G-100 Sephadex - 55 A_{260} units applied.

Distribution - peak 1 - 8%, peak 2 - 92%.


a.
A_{260}
2
1
2
total nucleic acid.
b.
A_{260}
2
1
high MW. RNA + DNA.
c.
A_{260}
2
1
2
tRNA
20
40
60
80
fraction #

2 BD-cellulose chromatography of other aminoacyl-tRNAs

Five different H.cutirubrum aa-tRNAs were chromatographed on BD-cellulose under elution conditions suitable for the resolution of met-tRNA species. Their elution profiles are shown in figures 32 - 36. The new tRNA preparation (page 18) and the BD-cellulose used for figure 6a (page 49) were employed for these chromatograms.

The results provided information relevant to future purification of these tRNA species, particularly $tRNA^{tryp}$ (figure 32), and they indicated that the peak 1 material, observed with met-tRNA chromatography (pages 46 & 49), was not an artifact of chromatography but was a feature of the old impure tRNA preparation.

Figure 32

^{14}C-trypt-tRNA on BD-cellulose

1.5 x 26 cm. column, at 4°C with a flow rate of 40ml./hr.

Applied : 1 x 10^4 cpm. sp. act. ^{14}C-trypt. 23 mC/mmole.

Stepwise elution as shown with all buffers containing 10mM $MgCl_2$.

50mM sodium acetate, pH 4.5 and 5mM 2-mercaptoethanol.

2ml. fractions.

Distribution : only one peak eluted with the ethanol purge.

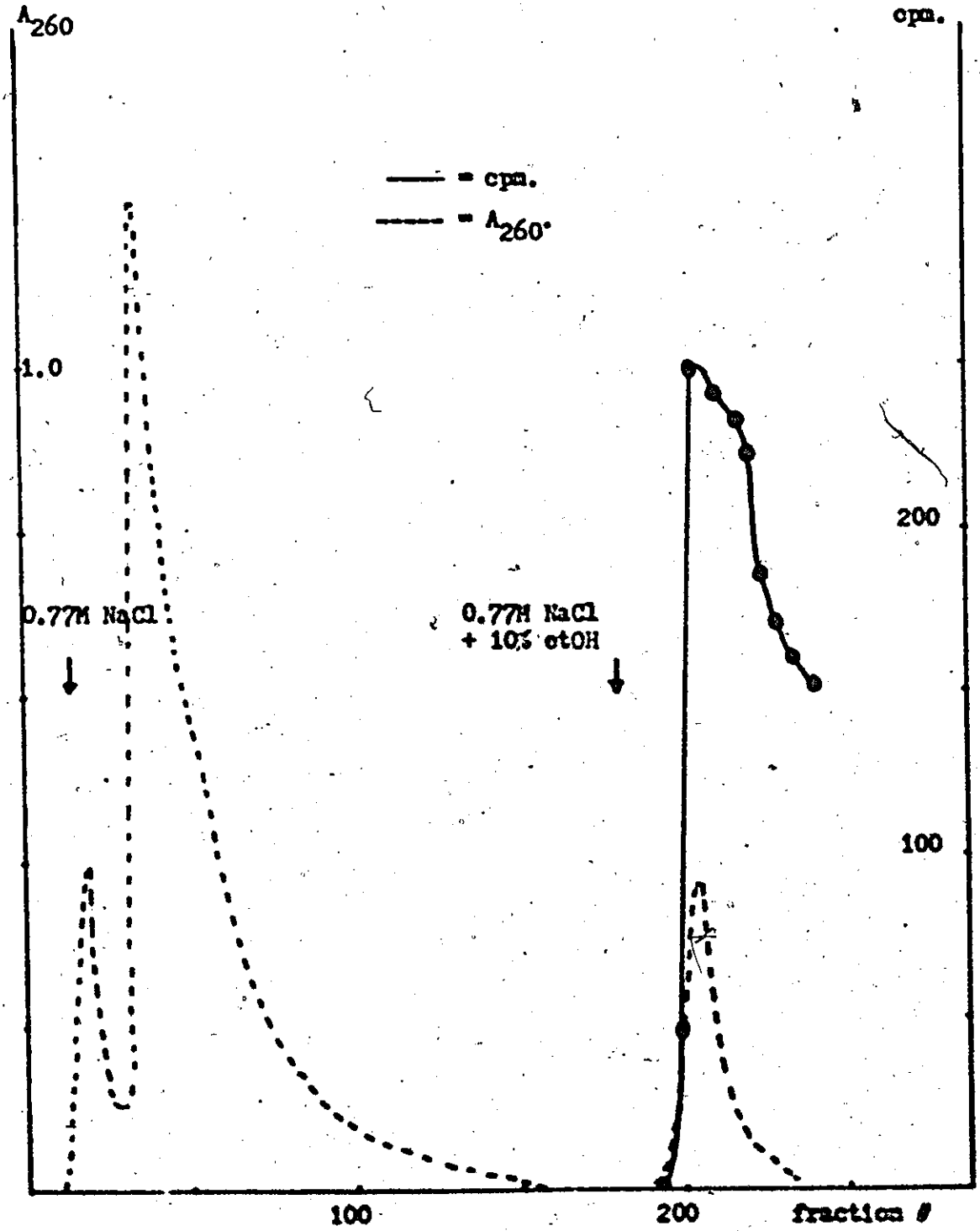

A_{260}
cpm.
—— = cpm.
—— = A_{260}.
1.0
200
100
0.77M NaCl
0.77M NaCl
+ 10% etOH
100
200
fraction #

Figure 33

^{14}C-val-tRNA on BD-cellulose

1.5 x 25 cm. column, at 4°C with a flow rate of 36ml./hr.

Applied : 2 x 10^6 cpm. sp. act. ^{14}C-val. 210 nC/mmole.

Stepwise elution as shown with all buffers containing 10mM $MgCl_2$, 50mM sodium acetate, pH 4.5 and 5mM 2-mercaptoethanol.

2ml. fractions.

Distribution : spread throughout chromatogram with possibility of at least three species.

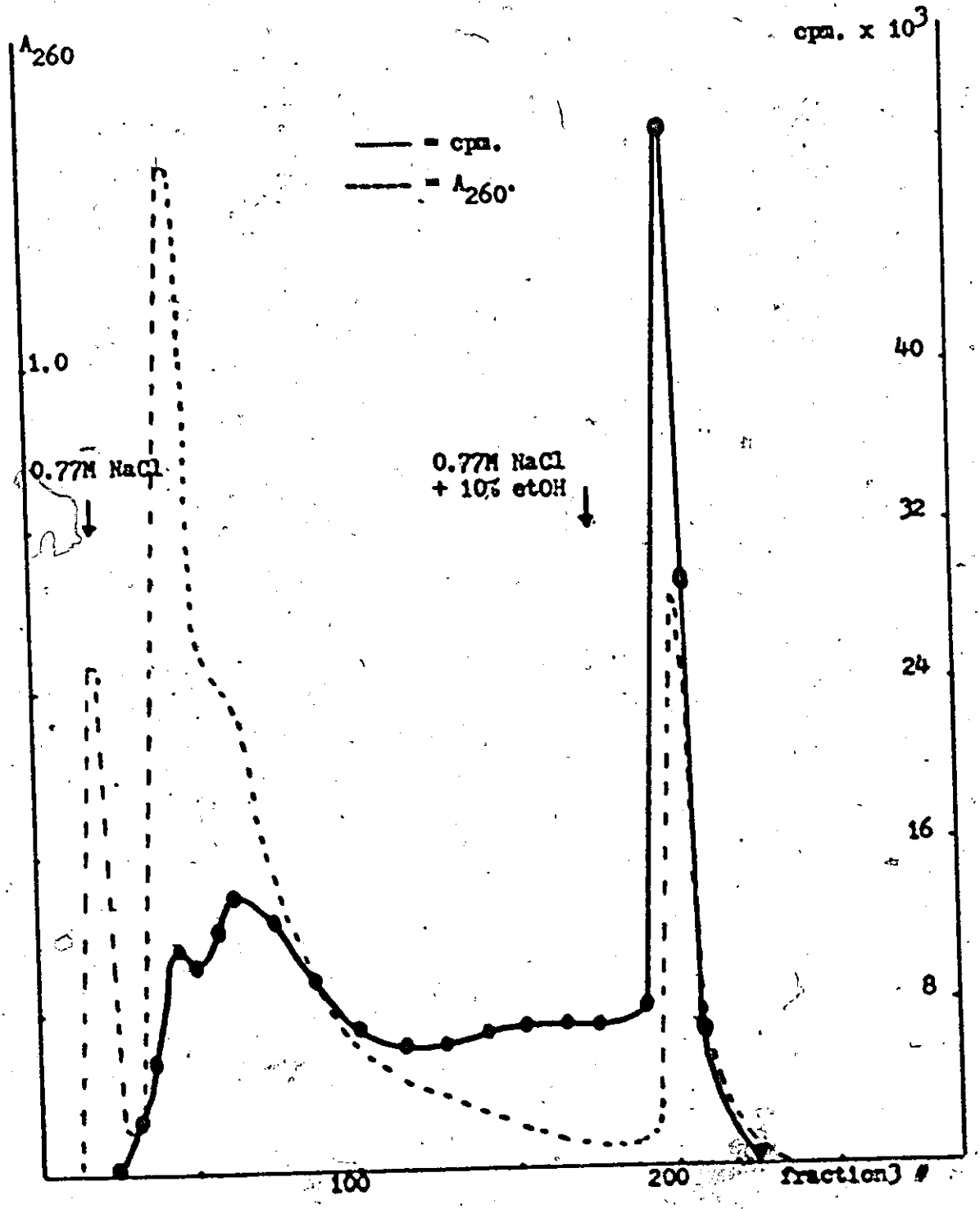

A_{260}
cpm. x 10^3
—— = cpm.
– – – = A_{260}.
1.0
0.77M NaCl
0.77M NaCl
+ 10% etOH
40
32
24
16
8
100
200
fraction #

Figure 34

^{14}C-glu-tRNA on BD-cellulose

1.5 x 25 cm. column, at 4°C with a flow rate of 36ml./hr.

Applied : 1.4×10^4 cpm. sp. act. ^{14}C-glu. 195 nC/mmole.

Stepwise elution as shown with all buffers containing 10mM $MgCl_2$.

2ml. fractions.

Distribution : three peaks; two in salt fraction and one in ethanol.

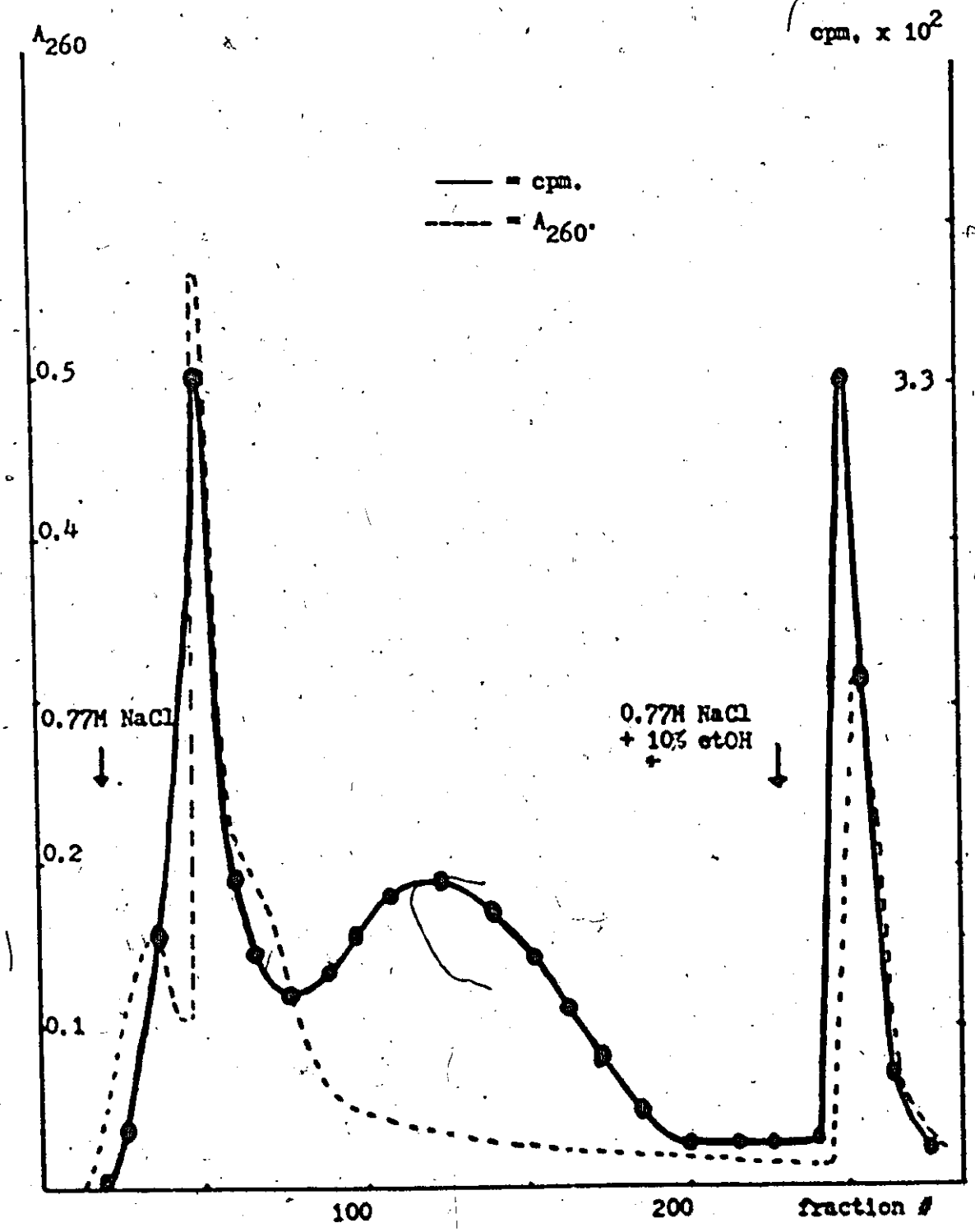

A_{260}
cpm, x 10^2
—— = cpm.
----- = A_{260}.
0.5
0.4
0.2
0.1
3.3
0.77M NaCl
0.77M NaCl
+ 10% etOH
100
200
fraction #

Figure 35

^{14}C-his-tRNA on BD-cellulose

1.5 x 25 cm. column, at 4°C with a flow rate of 36ml./hr.

Applied : 4 x 10^5 cpm. sp. act. ^{14}C-his. 239 nC/mmole.

Stepwise elution as shown with all buffers containing 10mM $MgCl_2$, 50mM sodium acetate, pH 4.5 and 5mM 2-mercaptoethanol.

2ml. fractions.

Distribution : one very sharp peak eluting with 0.77M NaCl flush.

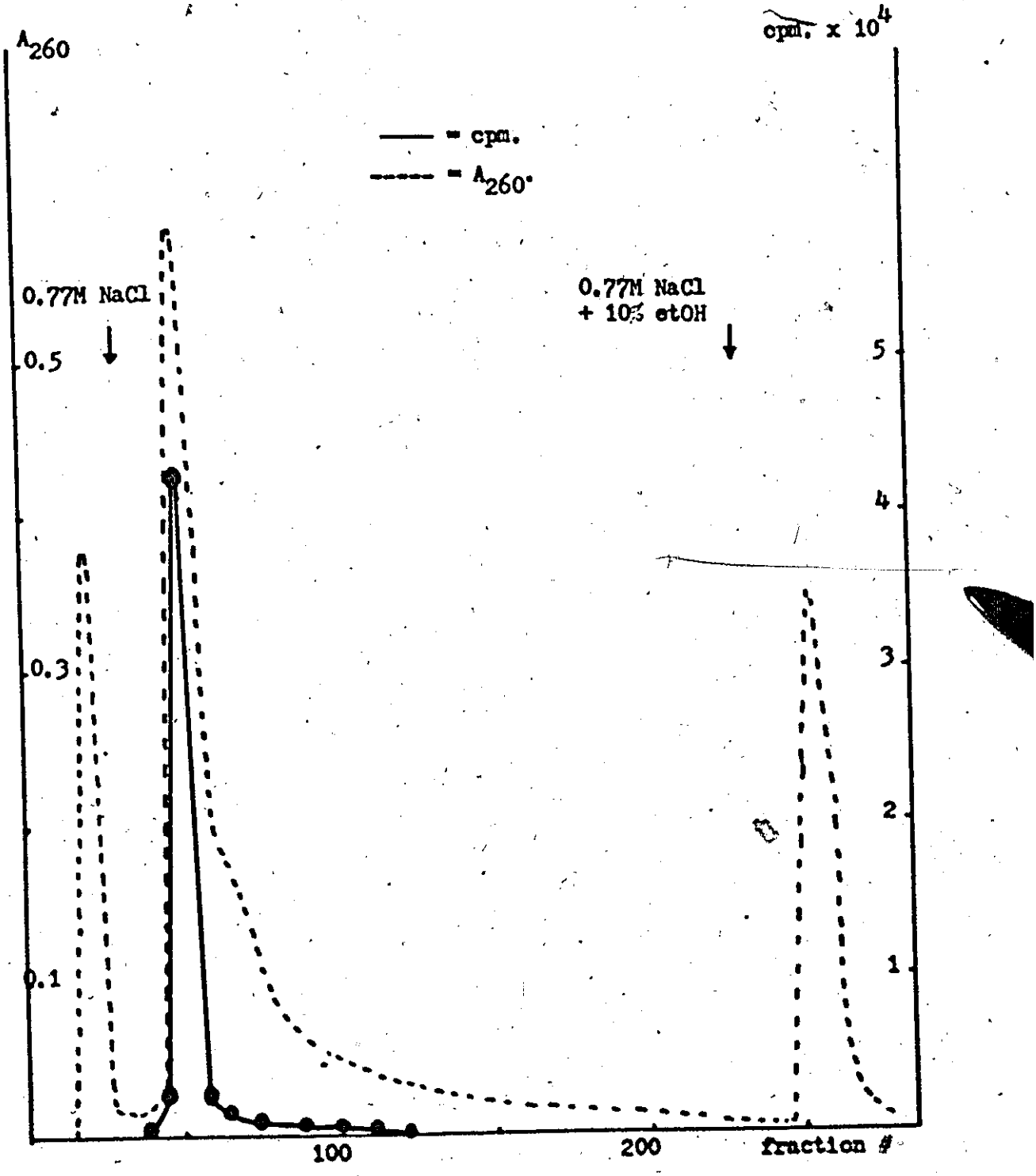

A_{260}
cpm. x 10^4
—— = cpm.
----- = A_{260}.
0.77M NaCl
0.77M NaCl
+ 10% etOH
0.5
0.3
0.1
5
4
3
2
1
100
200
fraction #

Figure 36

^{14}C-pro-tRNA on BD-cellulose

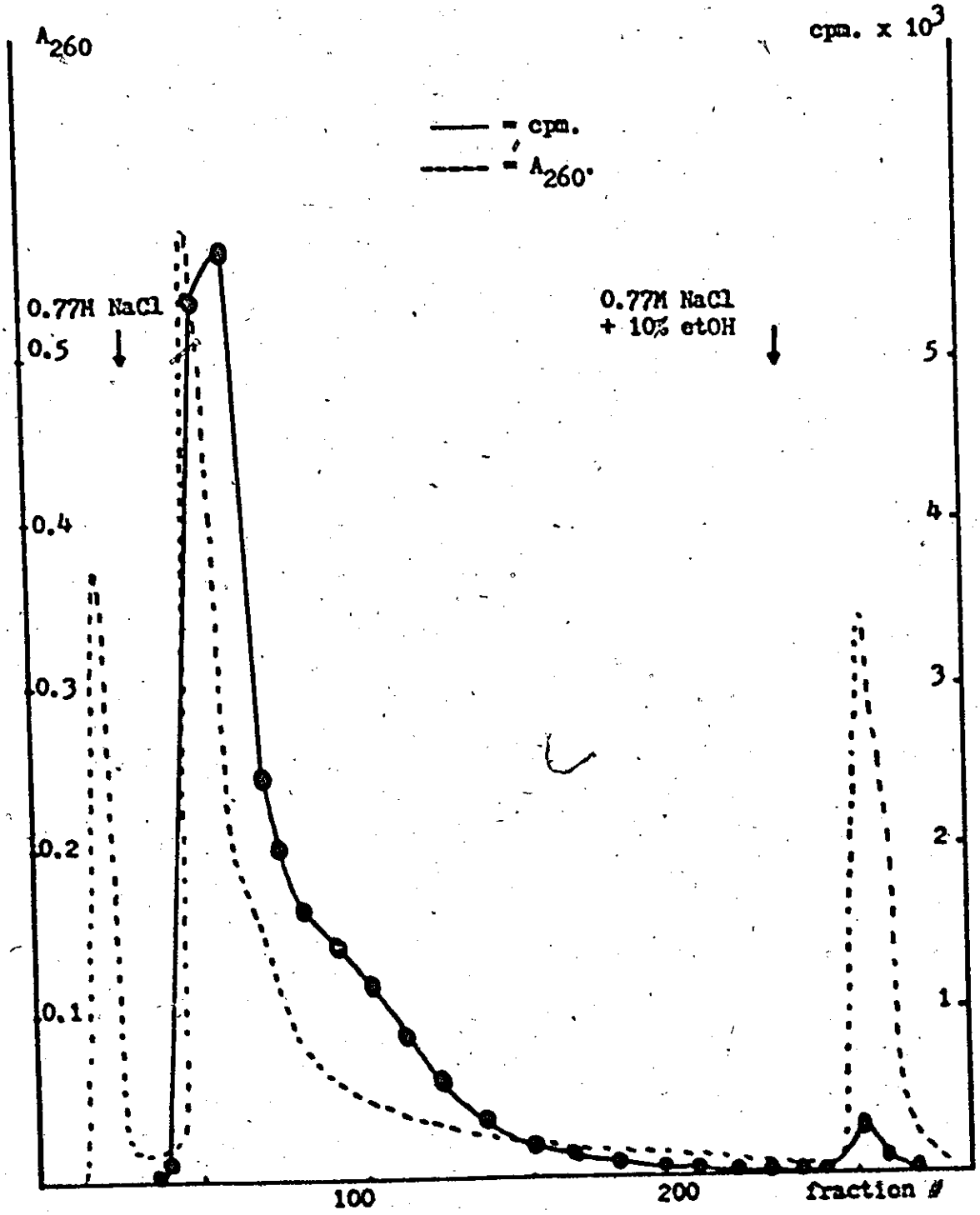
1.5 x 24 cm. column, at 4°C with a flow rate of 36ml./hr.

Applied : 2.6 x 10^5 cpm. sp. act. ^{14}C-pro. 209 mC/mmole.

Stepwise elution as shown with all buffers containing 10mM $MgCl_2$, 50mM sodium acetate, pH 4.5 and 5mM 2-mercaptoethanol.

2ml. fractions.

Distribution : one main peak with a shoulder eluting early in salt elution.


A_{260}
cpm. x 10^3
—— = cpm.
----- = A_{260}.
0.77M NaCl
0.77M NaCl
+ 10% etOH
0.5
0.4
0.3
0.2
0.1
5
4
3
2
1
100
200
fraction #