MECHANISMS IN PROTEIN SYNTHESIS IV. FURTHER EVIDENCE FOR TWO DIFFERENT RIBOSOMAL SITES, ONE BINDING FORMYLMETHIONYL-tRNA, THE OTHER METHIONYL- AND OTHER AMINOACYL-tRNA's.

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Methionine when esterified to one of the two methionine-accepting tRNA-species may become formylated (Marcker and Sanger,1964). The resulting Formylmethionyl-tRNA (FMet-tRNA) serves in the initiation of protein synthesis in E.coli (Adams and Capecchi, 1966; Webster et al., 1966). Compared to the synthesis of polyphenylalanine, the Mg^{++}-requirement for initiating cell-free polynucleotide-directed polypeptide synthesis is low in the presence of both FMet-codons and FMet-tRNA (Nakamoto and Kolakofsky, 1966). Apparently binding of (F)Met-$tRNA_{FMet}$ requires less Mg^{++} than binding Met-$tRNA_{Met}$ (Bretscher,1966). The binding activity of E.coli ribosomes for FMet-tRNA but not for most or all other species of aminoacyl-tRNA including the adapter for non-formylated methionine may be lost upon washing with 0.5 M ammonium chloride (Matthaei et al.,1966). We can strengthen and increase this evidence for the existence of two different ribosomal sites; (F)Met-$tRNA_{FMet}$ - whether formylated or not - is bound at around 4 and hardly above 10 mM Mg^{++}; Met-$tRNA_{Met}$, however, shows optimal binding near 16 mM and hardly below 5 mM Mg^{++}, thereby resembling other aminoacyl-tRNA-species. Washing ribosomes three times with ammonium chloride often completely removes the former activity,

whereas the latter remains. In binding the nonformylated mixture of methionine-adapters, the same two Mg^{++}-optima are seen.

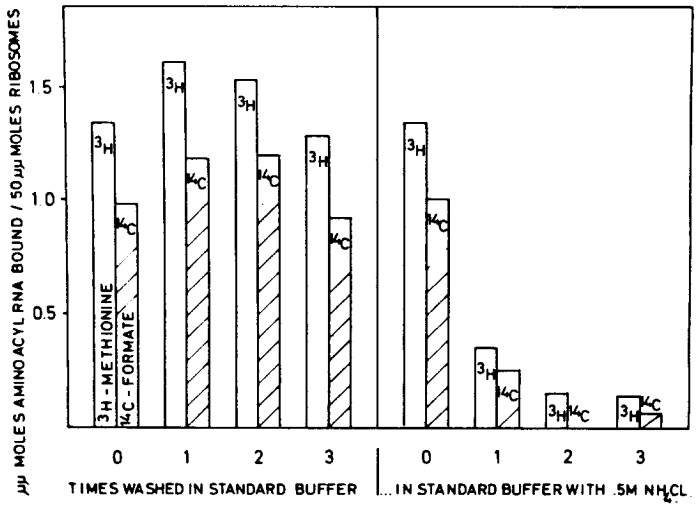

Fig. 1. Binding of FMet-tRNA to ribosomes washed previously in standard buffer or in standard buffer including 0.5 M NH_4Cl. The binding reaction was carried out in 100 µl of 30 mM Tris-HCl pH 7.2 - 6 mM KCl - 3 mM $MgCl_2$ (optimal Mg^{++}-concentration for binding FMet-tRNA in this experiment). 0.055 OD_{260}-units of polyA,U,G, 1200 µµmoles of ^{14}C-F-^{3}H-Met-atRNA*, and 50 µµmoles ribosomes** were incubated for 15 min. at 37°C. Samples were filtered with 10 mM Tris-HCl pH 7.2 - 10 mM $MgCl_2$ through Millipore-filters (HAWP 0.45 µ, 25 mm diameter), which were counted by scintillation in toluene containing 0.4 % PPO - 0.01 % POPOP. Minus mRNA-blanks are substracted.

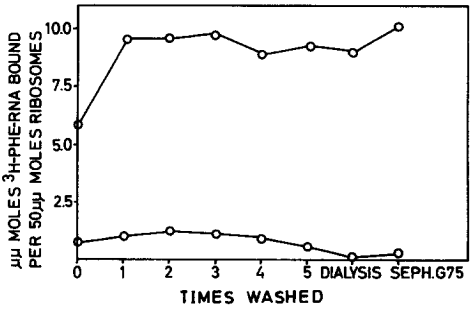

Fig. 2. Binding of ^{3}H-Phe-tRNA to ribosomes after various steps of purification. The lower curve gives the minus poly-U-blanks. Washing was carried out as described with 0.5 M ammonium chloride in standard buffer, dialysis for 20 h against 0.2 mM $MnCl_2$ - 0.1 mM EDTA - 10 mM Tris-HCl pH 7.4 at 4°C, sephadexing in the latter buffer. After washing 5 times and Mn-dialysis, RNase was 2 x 10^{-4} of original contamination (Voigt and Matthaei,1967).

*1 OD_{260}-unit=1.6mµmoles tRNA; **1 OD_{260}-unit=21µµmoles 70S-ribosomes.

METHODS. Ribosomes from E.coli A19 were prepared up to the P100 as described previously (Matthaei and Nirenberg,1961; Matthaei et al.,1967a). They were washed three times, either in standard buffer (10 mM Tris-HCl pH 7.2 - 60 mM KCl - 10 mM $MgCl_2$), or in 0.5 M ammonium chloride dissolved in standard buffer of pH 7.9; between the 3h-sedimentations at 134.000 x g (4^oC), ribosome suspensions were spun for 10 min. at 10.000 x g. tRNA from A19 was charged with 3 % ^{3}H-methionine (sp.act. = 1360 mc/mmole) and 19 non-labelled amino acids as before (Matthaei, et al.,1967a) and 1 % formylated with ^{14}C-formyltetrahydrofolate (sp.act. = 20 mc/mmole) and an S100 from E.coli A19. This product may be designated ^{14}C-F-^{3}H-Met-atRNA (cf. Schoech and Matthaei,1967). ^{3}H-Tyr- and ^{3}H-Phe-atRNA were synthesized in the same system, but not formylated. Poly-A,U,G was synthesized with a fraction III of polynucleotide phosphorylase from Micrococcus lysodeikticus (Matthaei et al.,1967a) at a substrate ratio of 1 ADP : 2 UDP : 1 GDP using ApU as primer. Conditions of the binding assay are indicated under Fig.1.

RESULTS. Whereas washing ribosomes three times in standard buffer has little effect on the binding of FMet-tRNA coded by poly-A,U,G , even one washing with 0.5 M ammonium chloride often substantially decreases this ribosomal activity (Fig.1). In contrast, this washing does not diminish the capability for binding phenylalanyl-, lysyl-, or prolyl-tRNA, but even increases it in the first step (Fig.2), paralleled by a higher activity of the ribosomes in the polyphenylalanine synthesizing system (Voigt and Matthaei,1967).

Figures 3 to 5 show that the Mg^{++}-optima for binding FMet-tRNA and Met-tRNA can be taken as further evidence for two different ribosomal sites: On once standard buffer-washed ribosomes, FMet-

tRNA, and no Met-tRNA was bound at 3 mM Mg^{++} (Fig.3). Stoichiometry in the binding of ^{14}C-formate and ^{3}H-methionine at 3 mM Mg^{++} will be seen in a subsequent communication (Matthaei and Milberg,1967), where special attention has been given to a maximal formylation of $Met\text{-}tRNA_{FMet}$. Whereas binding of $Met\text{-}tRNA_{Met}$ can hardly be realized below 5 mM Mg^{++}, this adapter binds optimally near 16 mM where, in turn, no FMet-tRNA is bound.

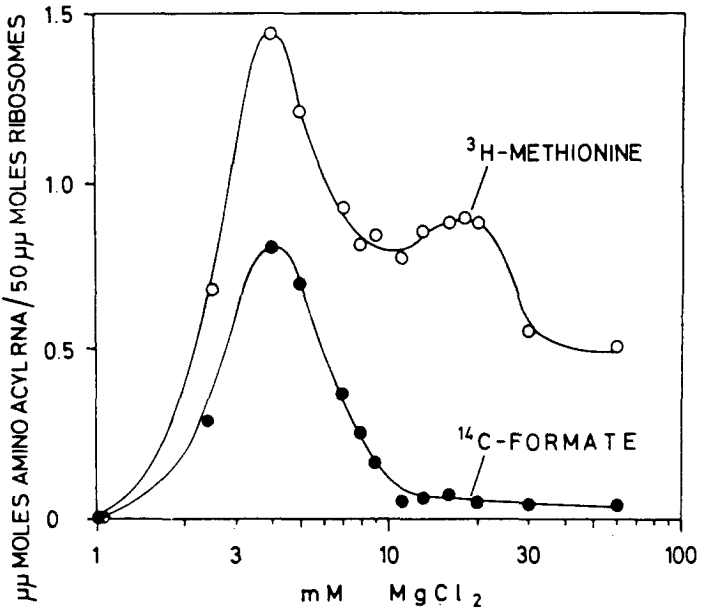

Fig.3. Binding of FMet- and Met-tRNA to once standard buffer-washed ribosomes; Mg^{++}-dependence. Assay conditions indicated under Fig.1. Mg^{++}-concentrations tested were used also for washing ribosomes on the filter.Fig.3-5:Minus-mRNA-blanks subtracted.

Binding of non-formylated ^{3}H-Met-tRNA displays the same two optima, indicating that $Met\text{-}tRNA_{FMet}$ binds also in the non-formylated state as shown by Bretscher (1966) and by Leder and Bursztyn (1966). Binding of $Met\text{-}tRNA_{FMet}$ is much more stimulated by K^{+} - ions at its Mg^{++} -optimum (3 mM) when in the formylated (+ 134%; Fig.6) rather than in the non-formylated state (+34%; Voigt and Matthaei,1967); this resembles the higher activity of

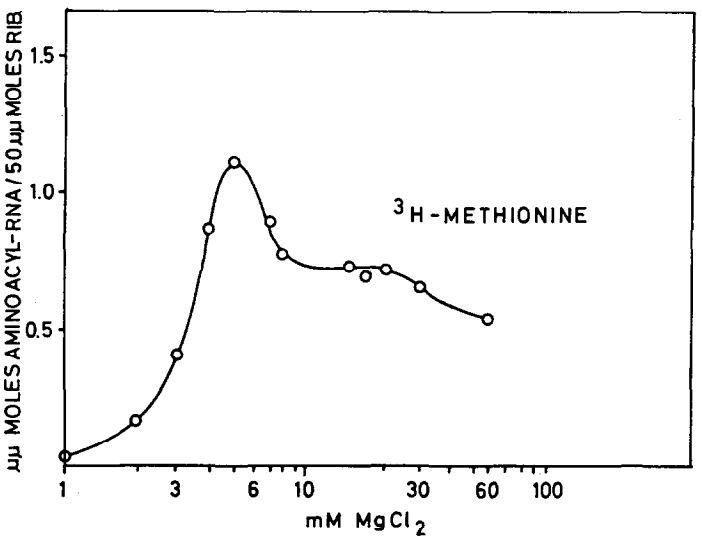

Fig.4. Binding of non-formylated ^{3}H-Met-tRNA to once standard buffer-washed ribosomes: Mg^{++}-dependence. Assay conditions indicated under Fig.1. Mg^{++}-concentrations tested were used also for washing ribosomes on the filter.

formylated Met-$tRNA_{FMet}$ in the transfer to puromycine (Bretscher, 1966). yin Fig.5,three-times ammonium chloride-washed ribosomes, which have lost the capacity to bind FMet-tRNA, still show the same optimum for binding Met-$tRNA_{Met}$;here, it is clearly seen that this chain-internal adapter hardly binds below 5 mM Mg^{++}.
This loss of capability to bind FMet-$tRNA_{FMet}$ has been shown to be very specifically restricted to this particular adapter (Matthaei et al.,1966). This loss does not seem to affect the codon-anticodon-recognition process (Matthaei and Milberg,1967). The similarity of the Mg^{++}-optima in binding Tyr- and Phe-tRNA due to other codons present in poly-A,U,G confirms the conclusion that Met-$tRNA_{Met}$ is bound to the same site (presumably the acceptor-site) (Fig.7,8).

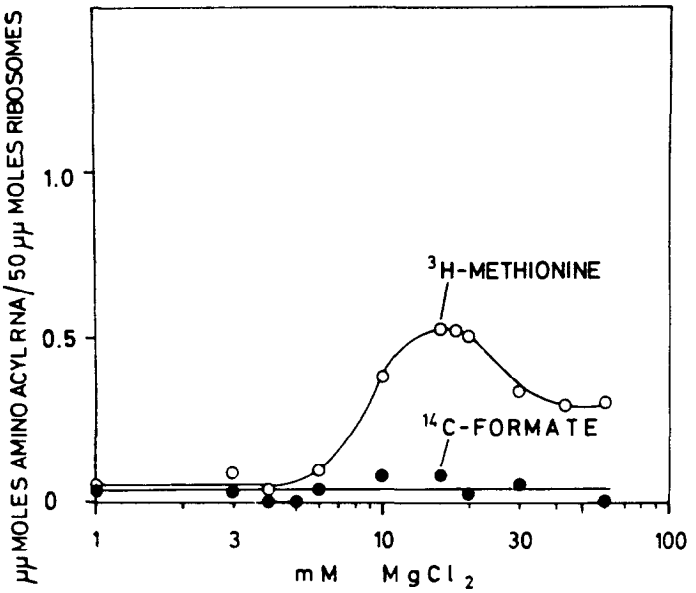

Fig. 5. Binding of ^{3}H-Met-tRNA and ^{14}C-F-^{3}H-Met-tRNA to three-times ammonium chloride-washed ribosomes: Mg^{++}-dependence. Assay conditions indicated under Fig. 1, except Mg^{++}-concentrations. Washing on the filter with 10 mM Tris-HCl pH 7.2 - 10 mM $MgCl_2$.

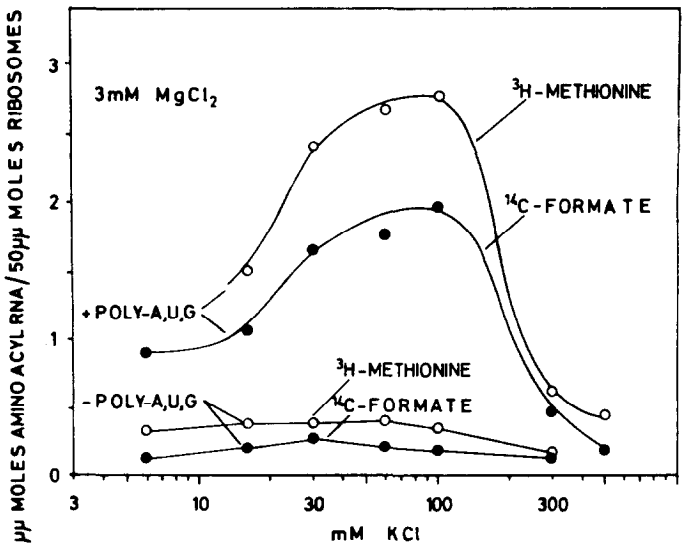

Fig. 6. Binding of ^{14}C-F-^{3}H-Met-tRNA to once standard buffer-washed ribosomes at 3 mM Mg^{++} (optimal in this experiment): Stimulation by K^{+}. Assay conditions indicated under Fig.1; washing on the filter with 10 mM Tris-HCl pH 7.2 - 3 mM $MgCl_2$.

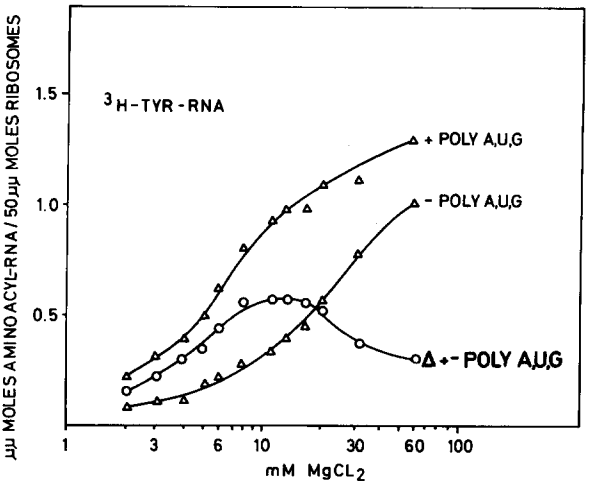

Fig. 7. Binding of ^{3}H-Tyr-tRNA to once standard buffer-washed ribosomes stimulated by poly-A,U,G: Mg^{++}-dependence. 100 μl-reaction mixtures contained 50 μμmoles ribosomes, 0.028 OD_{260}-units poly-A,U,G, and 600 μμmoles ^{3}H-Tyr-atRNA. For other assay conditions see Fig.1.

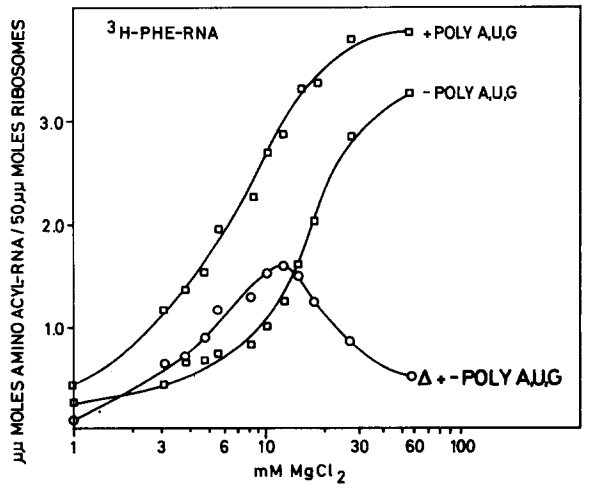

Fig. 8. Binding of ^{3}H-Phe-tRNA to once standard buffer-washed ribosomes stimulated by poly-A,U,G: Mg^{++}-dependence. Assay as in Fig. 7.

DISCUSSION. These and further results to be published will be discussed in order to find ultimately the sequence of events in which codons, and particularly FMet-codons, as constituents of mRNA, and likewise aminoacyl-tRNA and FMet-tRNA, enter the ribosomal sites. The two-site model, with an acceptor- or amino-acyl-tRNA-site and a donor-, peptidyl-RNA- or FMet-site is generally considered now. The possible existence of further sites for leaving (Wettstein and Noll, 1965) and maybe also for entering these two sites should be kept under consideration. Bretscher (1966) has used the isolated methionine-adapters $tRNA_{Met}$ and $tRNA_{FMet}$ and an assay for Met-puromycine to show that this peptide was derived essentially from the formylatable Met-adapter, which was bound at a lower Mg^{++}-concentration (10 mM at 100 mM Tris-HCl pH 7.1 - 50 mM NH_4Cl), whereas Met-tRNA was better bound at 20 mM Mg^{++}. The conclusion that (F)Met-$tRNA_{FMet}$ is bound to the donor-site, because transfer to puromycine is assumed to occur from it, is supported by further evidence presented in this series of observations.

It has been shown that both mRNA- and aminoacyl-tRNA-binding appear to be on the 30S-ribosomal subunits (Matthaei et al., 1964), whereas the transfer to puromycine occurs on the 50S-subunits (Monro, 1967). With the ribosome preparations and ionic conditions used here, the Mg^{++}-optima for binding (F)Met-$tRNA_{FMet}$ and Met-$tRNA_{Met}$ are not only different; they even mutually exclude the apparent binding of the other Met-adapter. The possibility that the (F)Met-$adapter_{FMet}$ might be bound at a different Mg^{++}-optimum, but to the same site as most amino acyl-tRNA's is contradicted further by the observation that, once bound at 3 mM Mg^{++}, it will not come off when this ribosomal complex is transferred to 16 mM Mg^{++} (Milberg and Matthaei, 1967). The

similarity of the Mg^{++}-optima for many aminoacyl-tRNA's and Met-$tRNA_{Met}$ suggests their binding to one common site, probably the acceptor site. This site appears to be directed by all 5'-terminal triplets in synthetic mRNA's of the type XpYpZ...pZ in binding all chain-internal amino acid-adapters to ribosomes (Matthaei et al.,1965) with the only exception of FMet-tRNA (Matthaei et al.,1966). These decoding experiments have been performed with five-times ammonium chloride-washed ribosomes. Although these generally do not bind FMet-tRNA upon the addition of ApUpI...$pI_{\sim 100}$ or poly-A,U,G , they still indicate the poly-U-directed binding of the Phe-adapter into the acceptor- and donor-sites by saturating equally two sites with Phe-tRNA (Matthaei and Milberg,1967). It therefore appears that ammonium chloride-washing does not remove the capacity for checking codon-recognition in both donor- and acceptor-sites, but rather detaches a factor (or factors) engaged in the binding of FMet-codons or their shift from acceptor- to donor-site or both. In preliminary experiments, refeeding the first ammonium chloride-wash enabled the ribosomes to bind again FMet-tRNA upon addition of poly-A,U,G. These may indicate the presence of the factor(s) studied by Stanley et al. (1966), Revel and Gros (1966), and Brawermann and Eisenstadt (1966), Whereas our random polymer poly-A,U,G and the nucleoside-diphosphate ApUpG (Bretscher,1966) apparently supply Met-adapter coding triplets to both acceptor- and donor-sites, the sequence of binding events with the model ApUpGpU...pU is under investigation.

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REFERENCES (more complete data and references in Voigt et al.1967).

Adams, J., and Capecchi, M. (1966), Proc.Nat.Acad.Sci. 55, 147.
Brawerman,G. and Eisenstadt,J.(1966), I.E.G. 7, No.420
Leder, P., and Bursztyn, H. (1966), Proc.Nat.Acad.Sci. 56, 1579.
Marcker, K., and Sanger, F. (1964), J.Mol.Biol. 8, 835.
Matthaei, H., Amelunxen, F., Eckert, K. und Heller,G (1964), Ber. Bunsenges. Phys.Chem. 68, 735.
Matthaei, H., Heller, G., Voigt, H.-P., Kleinkauf H., Küntzel, H., Vogt, M., and Matthaei, H., (1965), Naturw. 52, 653.
Matthaei, H., Heller, G., Voigt, H.-P., Neth, R., Schoech, G., and Kübler, H., (1967a), in: Genetic Elements (Academic Press, D.Shugar, ed.,) p. 233.
Matthaei, H., and Milberg, M. (1967), Biochem.Biophys.Res.Comm. (in preparation).
Matthaei, H. and Nirenberg, M.W. (1961), Proc.Nat.Acad.Sci.,47,1580.
Matthaei, H., and Obermeier, R.,(1967c), Unpublished results.
Matthaei, H., Voigt, H.-P., Heller, G., Neth, R., Schoech, G., Kübler, H., Amelunxen, F., Sander G., and Parmeggiani, A., (1966), Cold Spring Harbor Symposium on Quantitative Biology, Vol. XXXI, 25-38.
Milberg, M., and Matthaei, H. (1967). Unpublished results.
Monro, R.E. (1967), J.Mol.Biol. 26, 147.
Nakamoto, T., and Kolakofsky, D., (1966), Proc.Nat.Acad.Sci, 55,606.
Revel, M. and Gros, F. (1966), Biochem.Biophys.Res.Comm. 25, 124.
Schoech, G.K. and Matthaei, H. (1967), Hoppe Seyler's Z.Physiol. Chem. (in preparation).
Voigt, H., and Matthaei, H. (1967), Hoppe Seyler's Z. Physiol. Chem. (in press).
Webster, R.E., Engelhardt, D.L., Zinder, N.D. (1966), Proc.Nat. Acad.Sci. 55, 155.
Wettstein, F.O., and Noll, H. (1965), J.Mol.Biol. 11, 35.