chromasia was not limited to mammalian cells but occurred also in primitive eukaryotic organisms. A model is proposed which postulates that the cell membrane is permeable to the substrate, a nonpolar compound, and less permeable to the polar product, fluorescein. Quantitative studies of fluorochromasia with fluorescein analogues, both at the population and single cell level, are consistent with this model.

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[9] Subsequently, we have demonstrated that fluorescein can be forced to accumulate intracellularly when the external concentration is 10,000-fold higher than that used for fluorochromasia.

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## *DIRECTION OF READING OF THE GENETIC MESSAGE, II**

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It was shown previously[1] that in a cell-free system of protein synthesis consisting of purified *Escherichia coli* ribosomes and *Lactobacillus arabinosus* supernatant, a system of low nuclease activity, oligonucleotides of the type ApApApApApAp... pApApC[2] (AAAAAA...AAC) with an AAC codon at the 3′-end of the chain, directed the synthesis of oligopeptides of the structure Lys-Lys-Lys...Lys-Asn with $NH_2$-terminal lysine and COOH-terminal asparagine. These results showed (*a*) that AAC is an asparagine codon (AAA being a known codon for lysine), and (*b*) that if the biological assembly of the polypeptide chains of proteins proceeds, as currently believed, from the $NH_2$-terminal through the COOH-terminal amino acid, the genetic code is translated by reading the messenger from the 5′- to the 3′-end of the polynucleotide chain. The latter conclusion would be greatly strengthened if it could be shown that polynucleotides of the type AACAAA...AAA, containing the AAC codon at the 5′- rather than the 3′-end, directed the synthesis of lysine peptides with an asparagine residue at the $NH_2$-terminal end.

Oligonucleotides of the type ApApCpApApAp...pApApA ($A_2CA_n$) were synthesized and tested for lysine (AAA codon), asparagine (AAC codon), and threonine

(ACA codon[3]) incorporation in the *E. coli-L. arabinosus* system. However, these polynucleotides promoted the incorporation of negligible amounts of asparagine and threonine into tungstic acid-insoluble material relative to the amount of lysine incorporated. The low effectiveness of the ApApC triplet at the 5′-end could conceivably be due to the lack of a 5′-phosphate residue. In order to overcome this difficulty, oligonucleotides of the type ApApApApApCpAp...pApApA ($A_5CA_n$) and ApApApApCpAp...pApApA ($A_4CA_n$) were synthesized. The former ($A_5CA_n$) promoted the incorporation of lysine, asparagine, and small amounts of threonine, whereas the latter ($A_4CA_n$) promoted the incorporation of lysine, threonine, and traces of asparagine. Carboxypeptidase assays showed that the bulk of the asparagine in the lysine-containing oligopeptides synthesized with $A_5CA_n$ and the bulk of the threonine in those synthesized with $A_4CA_n$ messengers was in $NH_2$-terminal position. These results provide strong additional support for the conclusion that the genetic message is read in a 5′ → 3′ direction.

*Materials and Methods.*—These were as in a previous work[1] except as otherwise noted.

*Ribosomes and supernatant with low nuclease activity: E. coli* ribosomes purified by washing with ammonium chloride followed by chromatography on DEAE cellulose, and *L. arabinosus* supernatant were prepared as previously described.[1] It was important for the present experiments to determine whether these cell fractions had any 5′-exonuclease[4] activity. The virtual absence of endonuclease and 3′-exonuclease had already been ascertained but no specific assays for 5′-exonuclease had been carried out. For this purpose, oligonucleotides of the type A*pCpAp ... pApApA ($A^*CA_n$), with the adenine residue at the 5′-end labeled with $C^{14}$, were prepared. A sample (60 μg, 2500 cpm) of $A^*CA_{24}$ was incubated with purified *E. coli* ribosomes and *L. arabinosus* supernatant, under the usual conditions for amino acid incorporation, for 40 min at 37°. After adding 4 vol of cold ethanol, the precipitate was removed by centrifugation and the supernatant concentrated to a small volume under nitrogen. Samples were spotted on Whatman no. 1 paper and developed with 40% ammonium sulfate.[5] Areas corresponding to cyclic AMP, 2′-AMP, 3′-AMP, and adenosine were cut out and their radioactivity was determined. No radioactivity above that of paper blanks of corresponding location or zero time controls was found in any of the areas. From this it may be concluded that more than 99% of the 5′-termini of oligonucleotides of the kind used in this work remain intact during the usual conditions of protein synthesis in the purified *E. coli* ribosomes-*L. arabinosus* supernatant system.

*Preparation and characterization of oligonucleotides:* The preparation and characterization of the oligonucleotides used in this work will be described in detail elsewhere. The following is an outline of the procedures followed.

Poly $A^*CA_n$ and poly $A_2C^*A_n$, with the adenine and cytosine residues, respectively, labeled with $C^{14}$, were prepared with polynucleotide phosphorylase using ADP as substrate and either A*pC or ApApC* as primer. The polymers were isolated by exclusion chromatography on Sephadex G-100 at 25° in 8.0 *M* urea, 0.5 *M* ammonium bicarbonate, pH 8.6, and recovered by evaporation and lyophilization after exhaustive dialysis against distilled water. The primers were prepared by digestion of random poly A*C (1:1) or poly AC* (2:1) with pancreatic ribonuclease, phosphomonoesterase treatment, and size-fractionation by chromatography on DEAE cellulose in ammonium bicarbonate, pH 8.6.[6]

Poly $A_4C^*A_n$ and poly $A_5C^*A_n$, with the cytosine residue labeled with $H^3$, were prepared by the addition of one single cytidylic acid residue (from $H^3$-labeled CDP) to ApApApA ($A_4$) or ApApApApA ($A_5$) with polynucleotide phosphorylase in the presence of pancreatic ribonuclease, to form ApApApApC*p ($A_4C^*p$) or ApApApApApC*p ($A_5C^*p$). This was followed by removal of the 3′-terminal phosphate with phosphomonoesterase. The resulting $A_4C^*$ and $A_5C^*$, isolated by DEAE-cellulose chromatography in ammonium bicarbonate, were used as primers for the addition of adenylic acid residues from ADP with polynucleotide phosphorylase, and the resulting $A_4C^*A_n$ and $A_5C^*A_n$ polymers isolated as above by Sephadex G-100 chromatography. The $A_4$ and $A_5$, used as starting products, were prepared from poly A by hydrolysis in 0.1 *M* ammonium carbonate, pH 10, at 100° for an appropriate length of time, followed by acid hydrolysis of residual

cyclic phosphate ends, removal of the resulting terminal 2′- and 3′-phosphate residues with phosphomonoesterase, and chromatography on DEAE cellulose in ammonium bicarbonate.

A purified preparation of *Micrococcus lysodeikticus* polynucleotide phosphorylase, virtually free of nuclease and with a requirement for primer, was used for the addition of adenylic acid residues to A*C, $A_2C^*$, $A_4C^*$, and $A_5C^*$ primers, and for the addition of one ($H^3$-labeled) cytidylic acid residue to $A_4$, and $A_5$, essentially by the procedure of Thach and Doty.[7]

The various polymers were characterized by determining the location of the radioactivity following digestion with pancreatic ribonuclease. In each case all of the radioactivity was recovered in the species expected. $A^*CA_n$, $A_2C^*A_n$, $A_4C^*A_n$, and $A_5C^*A_n$ yielded A*Cp, $A_2C^*p$, $A_4C^*p$, and $A_5C^*p$, respectively. The average molecular weight and molecular weight distribution were determined both from the ratio of total nucleotide material (measured by absorbancy) to primer (determined by radioactivity), and from their chromatographic behavior on Sephadex G-100 previously calibrated with poly A, poly C, and poly U of known and uniform degree of polymerization. There was good agreement between the two methods indicating that all polymer chains were of the desired character and were free of poly (oligo) A chains. The chain lengths of the oligonucleotides used are given in Table 1.

TABLE 1

CHAIN LENGTHS OF OLIGONUCLEOTIDES ISOLATED BY CHROMATOGRAPHY ON SEPHADEX G-100

| Series | Polymer | Chain Length | |
|---|---|---|---|
| | | Average | Range |
| $A^*CA_n$ | $A^*CA_{\overline{24}}$ | 26 | 15–55 |
| $A_2C^*A_n$ | $A_2C^*A_{\overline{39}}$ | 42 | 30–65 |
| " | $A_2C^*A_{\overline{18}}$ | 21 | 15–26 |
| $A_5C^{**}A_n$ | $A_5C^{**}A_{\overline{19}}$ | 25 | 20–29 |
| " | $A_5C^{**}A_{\overline{10}}$ | 16 | 10–20 |
| $A_4C^{**}A_n$ | $A_4C^{**}A_{\overline{29}}$ | 34 | 27–40 |
| " | $A_4C^{**}A_{\overline{19}}$ | 24 | 19–27 |
| " | $A_4C^{**}A_{\overline{11}}$ | 16 | 9–19 |

* $C^{14}$-label; ** $H^3$-label.

*Amino acid incorporation and isolation of peptides:* The incubations, measurement of amino acid incorporation, and isolation of the peptides were as previously described.[1] The specific radioactivity of the $C^{14}$-lysine was 2 $\mu c/\mu mole$; that of the $C^{14}$-asparagine, 30 $\mu c/\mu mole$; and that of the $C^{14}$-threonine, 46 $\mu c/\mu mole$. In the amino acid incorporation assays (Table 1), 5 $\mu moles$ of nonlabeled amino acid (the one that was labeled in each particular incubation mixture) were added to each sample after incubation. Since the oligonucleotide messengers used contained in some cases $C^{14}$-cytidylic acid residues, the oligonucleotides in experiment 1 were then hydrolyzed, along with aminoacyl (or peptidyl) $\sim$ tRNA linkages, by further incubation for 16 hr at 37° with 0.5 *N* KOH, thus rendering the nucleotide radioactivity acid-soluble. In experiment 2, in which all of the oligonucleotides used had a $H^3$-labeled cytidylic acid residue, the samples (0.25 ml) were further incubated, as in previous work,[1] with 0.5 ml of 3% $NH_3$ for 1 hr at 37°. In all cases samples with labeled oligonucleotide, but without labeled amino acid, were run simultaneously with the experimental samples to correct for any radioactivity that might be contributed by the polymer.

*Results.—Amino acid incorporation:* The effect of the various oligonucleotides and, as a control, that of random poly AC (15:1) on the incorporation of lysine (AAA codon), asparagine (AAC codon), and threonine (ACA codon) is shown in Table 2. The incorporation of glutamine was not investigated because, as previously noted,[1] the *L. arabinosus* supernatant has low glutaminyl $\sim$ tRNA synthetase activity. All the polymers promoted the incorporation of lysine.

Poly AC promoted, as expected, the incorporation of equal amounts of asparagine and threonine. On the other hand, relative to lysine incorporation, only insignificant amounts of asparagine and threonine were incorporated with $A_2CA_n$ oligonucleotide messengers. As already pointed out, the presence of a terminal

TABLE 2

AMINO ACID INCORPORATION WITH POLY AC AND $(Ap)_nCpAp...pA$ OLIGONUCLEOTIDES

| Polynucleotide | Amino Acid Incorporation: μμmoles/Sample: Lysine | Asparagine | Threonine | Per cent of Total: Asparagine | Threonine |
|---|---|---|---|---|---|
| Expt. 1 | | | | | |
| None (blank)* | (115) | (23) | (69) | | |
| $A_2CA_{\overline{39}}$ | 2032 | 20 | 16 | 1.0 | 0.8 |
| $A_2CA_{\overline{18}}$ | 456 | 5 | 5 | 1.1 | 1.1 |
| $A_5CA_{\overline{19}}$ | 671 | 65 | 18 | 8.6 | 2.4 |
| $A_5CA_{\overline{10}}$ | 302 | 35 | 6 | 10.2 | 1.7 |
| Expt. 2 | | | | | |
| None (blank)† | (61) | (30) | (19) | | |
| Poly AC (15:1)* | 8151 | 912 | 848 | 9.2 | 8.6 |
| $A_5CA_{\overline{19}}$* | 954 | 95 | 20 | 8.9 | 1.9 |
| $A_5CA_{\overline{10}}$ | 445 | 72 | 8 | 13.7 | 1.5 |
| $A_4CA_{\overline{29}}$ | 1221 | 2 | 42 | 0.2 | 3.3 |
| $A_4CA_{\overline{19}}$‡ | 725 | 3 | 36 | 0.4 | 4.7 |
| $A_4CA_{\overline{11}}$ | 298 | 1 | 10 | 0.3 | 3.2 |

Conditions as described under *Amino acid incorporation*. Actual incorporation values (blanks without added polynucleotide subtracted from values with polynucleotide) expressed in μμmoles/sample. The cytidine residue in poly $A_2CA_{39}$ and poly $A_2CA_{\overline{18}}$ was labeled with $C^{14}$, that in $A_5CA_n$ and $A_4CA_n$ oligonucleotides was labeled with $H^3$.

* Average of duplicate runs.
† Average of six runs.
‡ Average of triplicate runs.

phosphate residue may be essential for reading of the ApApC triplet at the 5′-end of the chain.

Oligonucleotides of the $A_5CA_n$ series directed the incorporation of asparagine but threonine was incorporated to a much lesser extent. This result, suggesting that the artificial messengers are not read randomly, prompted the preparation and testing of $A_4CA_n$ oligonucleotides. The results were quite conclusive, as these polymers directed the incorporation of threonine but only traces of asparagine.

It may be noted that, relative to the incorporation of lysine, the incorporation of threonine in the experiments with $A_4CA_n$ oligonucleotides was substantially lower than that of asparagine in those with $A_5CA_n$ polymers of similar chain length. No explanation for this discrepancy can be offered at present. As previously reported,[1] the *E. coli-L. arabinosus* system does not cleave $C^{14}$-labeled heptalysine. However, presence of a threonine aminopeptidase activity would account for the observed results.

*Position of asparagine and threonine in peptide chains:* Peptides containing $C^{12}$-lysine and either $C^{14}$-asparagine or $C^{14}$-threonine were prepared with $A_5CA_{\overline{19}}$ and $A_4CA_{\overline{29}}$ messengers, respectively, and isolated by carboxymethyl-cellulose chromatography as previously described.[1] As shown in Figure 1, after treatment with carboxypeptidase A the distribution of the peptides containing $C^{14}$-threonine or $C^{14}$-asparagine remained essentially unchanged. This result indicates that neither of these amino acids was in COOH-terminal position in the lysine peptides for, as shown previously,[1] COOH-terminal asparagine is rapidly released from lysine peptides by carboxypeptidase A. Carboxypeptidase A is also known to hydrolyze off COOH-terminal threonine.[8] On the other hand, treatment with carboxypeptidase B resulted in the release of about 75 per cent of the radioactivity in each of the two peptides as free $C^{14}$-threonine or $C^{14}$-asparagine. Previously,[1] it had been shown that carboxypeptidase B has no effect on the size distribution of lysine peptides with COOH-terminal asparagine, and the same is to be expected of lysine

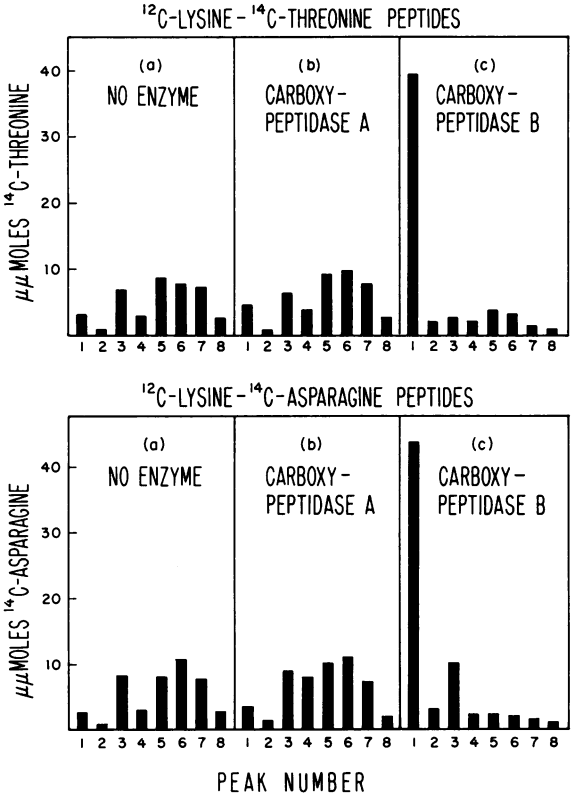


FIG. 1.—Effect of carboxypeptidases A and B on $C^{12}$-lysine-$C^{14}$-threonine and $C^{12}$-lysine-$C^{14}$-asparagine peptides. Peptides containing 56 μμmoles of $C^{14}$-threonine or 60 μμmoles of $C^{14}$-asparagine, prepared with $A_1CA_{\overline{29}}$ or $A_5CA_{\overline{19}}$ oligonucleotide messengers, respectively, and isolated as previously described,[1] were fractionated by chromatography on carboxymethylcellulose before (*a*) or after incubation for 30 min at 37° with either 13 μg of carboxypeptidase A (*b*) or 1.8 μg of carboxypeptidase B (*c*). The procedures for carboxypeptidase treatment and chromatography have been described.[1] The effluent was monitored continuously at 220 mμ. Fractions, 0.85 ml, eluted from the column were collected and their radioactivity was measured. Free threonine or asparagine, not retained by the column, are recovered in peak *1*. The succeeding peaks, *2*, *3*, etc., correspond to the dipeptide (1 thr or 1 asn, 1 lys), tripeptide (1 thr or 1 asn, 2 lys), etc., respectively. Free lysine would be recovered in peak *2*.

peptides with COOH-terminal threonine, for carboxypeptidase B requires COOH-terminal basic amino acids for activity.[9] The above results, therefore, indicate that the bulk of the asparagine and threonine in the lysine peptides investigated was in $NH_2$-terminal position.

*Discussion.*—The results presented in this paper provide conclusive evidence for the 5′ → 3′ polarity of translation of the genetic message. Recent results of other investigators[10, 11] are in accord with this view. The fact that (*a*) $A_2CA_n$ polymers promoted but negligible asparagine incorporation, and (*b*) that most of the asparagine and threonine, incorporated along with lysine in the incubations with $A_5CA_n$ and $A_4CA_n$ oligonucleotides, was in $NH_2$-terminal position, indicates that reading of the initial triplet at the 5′-end of the chain was frequently missed, possibly for lack of a terminal phosphate. Nirenberg and Leder[12] have noted that the presence of a 5′-phosphate increased the effectiveness of trinucleotides in

promoting specific binding of aminoacyl ~ tRNA to ribosomes. Triplets without terminal phosphate at the 5′-end of synthetic homopolynucleotides have been reported to promote specific binding of aminoacyl ~ tRNA to *E. coli* ribosomes.[13] In view of our results, it must be assumed that such binding would not, or would only infrequently, lead to reading of the triplet.

Our results may be interpreted to mean that, irrespective of the occurrence of actual reading leading to amino acid incorporation, translation of the oligonucleotide messengers starts at the 5′-end and that this start sets the reading frame (cf. Table 3). Faulty starts due to "jumping" one or two bases and resulting in shifts of

TABLE 3

READING OF $A_2CA_n$, $A_5CA_n$, AND $A_4CA_n$ OLIGONUCLEOTIDES

| Oligonucleotide | | Frame setting | Peptides synthesized |
|---|---|---|---|
| Poly $A_2CA_n$ | (*a*) | ↑ ApApC pApApA pApApA... | Lys-Lys-Lys...Lys; probably traces of Asn-Lys-Lys...Lys |
| | (*b*) | A ↑ pApCpA pApApA pApApA... | Probably traces of Thr-Lys-Lys...Lys |
| Poly $A_5CA_n$ | (*a*) | ↑ ApApA pApApC pApApA... | Asn-Lys-Lys...Lys; probably traces of Lys-Asn-Lys...Lys |
| | (*b*) | A ↑ pApApA pApCpA pApApA... | Probably small amounts of Lys-Thr-Lys ...Lys |
| Poly $A_4CA_n$ | (*a*) | ↑ ApApA pApCpA pApApA... | Thr-Lys-Lys...Lys; probably traces of Lys-Thr-Lys...Lys |
| | (*b*) | ApA ↑ pApApC pApApA... | Probably traces of Asn-Lys-Lys...Lys |

The vertical arrows mark the initial point of attachment of ribosomes to the messenger. The underlined triplets at the 5′-ends, lacking a terminal phosphate, are rarely read. "Jumping" of one or two bases with ensuing frame shift (as in (*b*) series of frame settings) appears to occur infrequently.

reading frame (as indicated in the (*b*) series of frame settings of Table 3) occurred rarely. It should be noted that these shifts must be the result of base "jumping" rather than that of enzymatic removal of 5′-terminal residues from the messengers for, as already pointed out, the cell fractions used in this work were devoid of 5′-exonuclease activity.

*Summary.*—Synthetic oligonucleotides of the type ApApApApApCpAp... pApApA ($A_5CA_n$) and ApApApApCpAp...pApApA ($A_4CA_n$) have an ApApA triplet at the 5′-end, followed by the asparagine codon pApApC in the $A_5CA_n$ series, or by the threonine codon pApCpA in the $A_4CA_n$ series. These are followed in both series by a number of pApApA (lysine) codons. In a system of purified *E. coli* ribosomes and *L. arabinosus* supernatant, of low nuclease content, $A_5CA_n$ oligonucleotides directed mainly the synthesis of lysine oligopeptides with $NH_2$-terminal asparagine, whereas $A_4CA_n$ polymers directed predominantly the synthesis of lysine oligopeptides with $NH_2$-terminal threonine. The initial ApApA triplet in these polymers was thus infrequently read. Together with earlier work[1] these results provide conclusive evidence for the 5′ → 3′ polarity of messenger translation. They also show that the above oligonucleotides are not read randomly by the cell-free system.

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## *N-FORMYLMETHIONYL-sRNA AS THE INITIATOR OF PROTEIN SYNTHESIS*

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The work several years ago of J. P. Waller[1] revealed a bizarre fact about N-terminal groups of bacterial proteins. Instead of a random mixture, Waller found that the great majority of N-terminal groups were either methionine or alanine. This finding suggested that methionine and alanine constituted start signals for the initiation of polypeptide chains. Alternatively, the remaining amino acids were not detected because of acylation of their amino groups. The discovery of N-formylmethionyl-sRNA by Marcker and Sanger[2] provided a means for further study of the problem.

In this compound, the amino group of methionine is formylated, thereby prohibiting its use in polypeptide chain elongation and at the same time making it an attractive candidate for initiation of polypeptide chains. Knowing of Waller's observations, Marcker and Sanger also looked for N-formylalanyl-sRNA. None, however, was detected. If chain initiation required formyl amino acids, the terminal alanine end groups of *E. coli* proteins remained unexplained. There was, of course, the possibility that N-formylmethionine was unrelated to protein synthesis.

The direct way to test involvement of N-formylmethionyl-sRNA in chain initiation is to add this compound to *in vitro* extracts which are carrying out protein synthesis. Here we report experiments in which N-formylmethionyl-sRNA