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*MEMBRANE PROPERTIES OF LIVING MAMMALIAN CELLS AS STUDIED BY ENZYMATIC HYDROLYSIS OF FLUOROGENIC ESTERS**

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Fluorogenic substrates are compounds which yield fluorescent products after enzymatic modification. Their utility for quantitative measurements of enzymatic activity at the cellular[1] and molecular level[2, 3] has been previously demonstrated. Among this group of substrates certain fluorescein esters of fatty acids appeared to be of particular interest for the study of living mammalian cells, since their hydrolysis resulted in the intracellular accumulation of fluorescein. In this report we provide evidence which indicates that the intracellular retention of fluorescein is dependent on the integrity of the cell membrane.

Materials and Methods.—Unless otherwise specified, all the experiments were performed with the 2B2 cloned subline[4] of the cultured mouse lymphoma, ML-388, which was kindly supplied by Dr. L. A. Herzenberg. Other cell lines included the human strains, HeLa no. 64266, Hep-2 no. 64267, and HESM no. 64269, a diploid fibroblast line isolated from embryonic skin and muscle. These were supplied by Tissue Culture Associates, Oakland, California.

Primary isolates of tissue pieces, or cell suspensions were obtained from chick embryos, newly hatched chicks, and adult mice. Peripheral blood cells from humans, mice, and chicks were also examined. Among primitive eukaryotic organisms, *Tetrahymena pyriformis, Dictyostelium discoideum* (kindly supplied by Drs. D. S. Natchtwey and R. Sussman, respectively), and isolates from pond water were studied.

Monolayer cultures of 2B2 were grown in a modified Eagle's MEM medium[4] with Earle's balanced salt solution (Microbiological Associates). Cells were removed from culture bottles by replacing the growth medium with 1 ml of phosphate buffered saline (PBS)[5] containing 10^{-4} *M* EDTA and 0.05% trypsin and incubating for 10 min at room temperature (23°C).

With the exception of fluorescein diacetate[6] (synthesized in this laboratory) and of fluorescein dibutyrate (Eastman Kodak), all other fluorescein derivatives[7] were prepared in Dr. J. Moffatt's laboratory at the Syntex Institute of Molecular Biology. For most compounds, a stock solution containing 5 mg per ml was made up in acetone and kept in the freezer. Prior to their use, a dilution of $1:10^4$ was prepared in aqueous medium. Solutions containing more than 1 μg per ml tended to flocculate. The appearance of fluorescence after addition of a fluorogenic substrate to cell suspensions or extracts was followed quantitatively at 37° in a thermoregulated Turner fluorometer model 111 using the filters BG-12 and Wratten 2A-12 for the exciting and fluorescent light, respectively. Purified fluorescein[8] was used as a reference standard.

The fluorescence of individual cells was observed under dark field using a Zeiss microscope equipped with a III Z condenser which permitted alternative observations with either dark field, bright field, or phase contrast. The ordinary tungsten lamp provided with the microscope, in conjunction with an interference filter transmitting a broad band (440–480 mμ), was used as the

source of exciting light. No secondary filter was used. For quantitative measurements,[2] a BG-12 was substituted for the interference filter and a Wratten 15 was added as secondary filter. An Aminco photomultiplier microphotometer mounted on the microscope served to measure the fluorescent light.[2]

Results.—Characterization of the fluorochromatic reaction with fluorescein diacetate in cultured lymphoma cells (2B2 line): Cells removed from the culture bottle with 1 ml of the EDTA-trypsin solution were resuspended in 10 ml of PBS to a concentration of about 10^6 per ml. This suspension was mixed on a microscope slide with a solution of fluorescein diacetate (FDA) in PBS to give a final concentration of about 0.1 μg of FDA per ml. An immediate reaction, characterized by the appearance of a bright-green fluorescence inside the cell, was observed when the preparation was examined under dark field with the appropriate light filters. We have termed this reaction *fluorochromasia.* Fluorescein at the same or 50-fold higher concentration produced no fluorochromasia.[9] Other fluorogenic substrates[8] such as 3-0-methylfluorescein acetate, 3-0-methyl-fluorescein phosphate, and fluorescein diphosphate, which were hydrolyzed by the cells yielding fluorescent products, did not produce the fluorochromatic reaction.

Chemical nature of the intracellular fluorescent products: Cells were incubated (23°) with FDA for 1 min, centrifuged down, and then extracted with methanol. A control, in which FDA was added after the addition of methanol, was run concurrently. The methanolic extract was chromatographed on thin-layer silica plates using 2 per cent methanol in chloroform as solvent. The fluorescence of the compounds was visualized after spraying with 1 *M* alcoholic KOH. In this system, fluorescein remains at the origin while fluorescein monoacetate and fluorescein diacetate move with Rf of 0.2 and 0.9, respectively. The results of this experiment indicated that fluorescein is the only product accumulated in the cells.

Kinetic studies: Cells were removed from the culture bottle with EDTA-trypsin and diluted 1:10 in PBS containing FDA at different concentrations. A drop of the cell suspension was put onto a microscope slide and a cover slip with the edges coated with silicone grease placed over it. The preparation was examined under the microscope and single cells were selected at random for quantitative measurement of fluorescence as indicated in *Materials and Methods.* As shown in Figure 1, the accumulation of intracellular fluorescein increased linearly with time. At the lowest concentration of FDA (6×10^{-8} *M*) used, the linear rate continued for a period of 50 min. The rate of fluorescein accumulation increased with substrate concentration according to Michaelis-Menten enzyme kinetics. The maximal rate of FDA hydrolysis, at saturating substrate concentration,

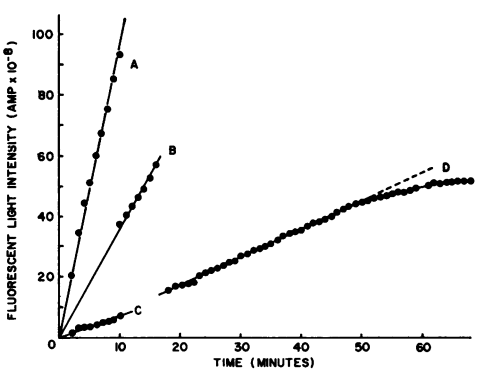


FIG. 1.—Kinetics of fluorescein accumulation in single cells. The substrate concentration was 6×10^{-7} (*A*), 3×10^{-7} (*B*), and 6×10^{-8} *M* FDA (*C*, *D*). Curves *C* and *D* represent kinetics of two different cells. The instrument was calibrated by measuring the fluorescence of droplets of 10.3-μ diameter containing 10^{-5} *M* fluorescein[2] which gave average readings of 36 fluorescent units. The values in the figure were not corrected for the background fluorescence of the intercellular space (less than one unit).

was about the same for intact cells and extracts. The K_m (concentration of substrate giving half the maximal rate) of the intact cells (2.9×10^{-6} M), however, was ten times lower than that of the cell-free extract (3.6×10^{-5} M).

The distribution of fluorochromatic activity among the cell population was studied by measuring the intracellular fluorescein accumulated in different cells after a period of 10 min incubation at room temperature (23°) in PBS containing 1.2×10^{-7} M FDA. Cells of about 20-μ diameter were selected. The amount of fluorescence varied extensively from cell to cell ranging up to 80 fluorimeter units. However, the majority (92%) exhibited values above 10 units.

Effect of pH and temperature: For the experiments on pH, the cells at a density of 10^5 per ml were incubated for 10 min in 0.25 ml of PBS, previously adjusted to the desired pH. The reaction, stopped by adding 0.75 ml of cold PBS (final pH near 7.3), was measured in the fluorometer. A broad pH maximum, 6.7–8.0, was found.

The effect of temperature on the rate of fluorochromatic reaction was also studied in cell suspensions. The Q_{10} was 1.71 (20–30°) and 1.78 (30–40°). These values converted to energy of activation gave an average of 10,300 cal/mole.

The enzymatic activity of both cell suspensions and extracts was inactivated at 50°. Five minutes at this temperature caused a 30 per cent loss of activity in the cell-free extract as compared with 60 per cent in the cell suspension. Heating for 30 min at 56° caused complete inactivation of both cells and extracts.

Specificity of the fluorochromatic reaction: A series of fluorescein esters was tested in order to determine the specificity involved in the fluorochromatic process (Table 1). For a disubstituted ester, the longer the chain length of the fatty acid, the less effective it was as a fluorochromatic substrate. If the acid contained more than 6 carbons, no fluorochromasia was observed. The ability of cell-free extracts to hydrolyze the esters was correlated with the fluorochromatic reaction of intact cells. Compounds not hydrolyzed by the extracts did not give fluorochromasia.

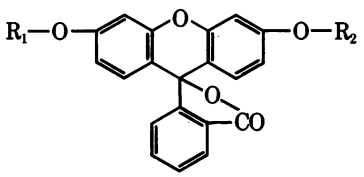
The monosubstituted esters were effective fluorochromatic substrates at a concentration of at least 10 times less than that of the disubstituted analogues. The K_m of fluorescein monoacetate, measured with cell suspensions, was 4×10^{-7} M.

Modifications of the fluorescein ring lead to a series of compounds with divergent properties (Table 1). The best illustration is the comparison between 3-0-methyl-fluorescein acetate and 2,7-dichlorofluorescein diacetate. The two compounds differ greatly in their fluorochromatic activity while exhibiting similar rates of hydrolysis by cell-free extracts. The results given in Table 2 demonstrate that the lack of fluorochromatic activity of 3-0-methyl-fluorescein acetate cannot be ascribed to absence of enzymatic activity in the intact cells. Both substrates, FDA and 3-0-methyl-fluorescein acetate, were hydrolyzed at the same rate by the cell suspension; however, less than one per cent intracellular accumulation of fluorescent product occurred with the latter substrate. Considering that known esterases do not have the specificity to differentiate between FDA and its methyl-ether acetate, the alternative possibility that 3-0-methyl-fluorescein acetate and FDA were hydrolyzed by different enzymes seems unlikely.

Kinetics of the exit process: The rate at which intracellular fluorescein was released into the medium was studied in single cells independently of the entrance

TABLE 1

FLUOROCHROMATIC ACTIVITY OF FLUORESCEIN DERIVATIVES



Acyl group	Fluorochromasia: Concentration (μmoles per liter)	Fluorochromasia: Fluorescence single cells	Extract Activity: Concentration (μmoles per liter)	Extract Activity: Rate (units per min)
Disubstituted ($R_1 = R_2$) R_2				
Acetyl	1.20	346	11.4	71
Acetyl	0.12	36	1.1	25
Propionyl	1.10	472	10.7	42
Butyryl*	1.10	150	3.8	50
Valeryl	1.00	26	9.5	6
Hexanoyl	1.00	2	9.0	1
Heptanoyl	0.90	2.4	8.5	0
Decanoyl	0.80	0.9	7.4	3
Dodecanoyl	0.70	0	6.8	1
Hexadecanoyl	0.60	0	5.9	0
Acrylyl	1.10	900	10.8	21
Methane sulfonyl	1.00	0	9.7	0
Isobutoxy carbonyl	0.90	6.7	8.9	2
Furoyl*	1.00	195	0.3	12
Benzoyl	0.90	0	8.8	0
Monosubstituted (R_1 = H) R_2				
Acetyl	0.13	430	0.02	9
Propionyl	0.12	330	0.05	26
Decanoyl	1.00	90	0.02	10
Ring modifications				
3-0-Methyl-fluorescein acetate	1.30	1.2	12.2	32
2,7-Dichloro-fluorescein diacetate	1.00	250	9.8	10
4,5-Dibromo-fluorescein diacetate	0.90	0	8.3	0

Fluorochromasia was determined in single cells after 20 min at 25° as outlined in the text. The values represent the average fluorescence of 20 cells (see Fig. 1 for calibration factor). The extracts were prepared by lysing the cell suspensions in a French press, sedimenting at 800 × *g* for 10 min and collecting the supernatant for the experiments. The activity of the extracts is given in Turner fluorometer units per minute. Under our conditions, 100 units correspond to a 7.81×10^{-8} *M* solution of fluorescein. The rates were corrected for the spontaneous hydrolysis rate of the substrates.

* This compound had some monoester as impurity.

mechanism. Cells were incubated with 4.8×10^{-7} *M* FDA for 20 min at room temperature (23°), centrifuged down, and resuspended in PBS. A drop of the cell suspension was placed on a slide, a cover slip (with the edges coated with silicone grease) dropped on it, and the fluorescence of any given cell was measured at intervals.

TABLE 2

COMPARISON BETWEEN FLUORESCEIN DIACETATE AND 3-0-METHYL-FLUORESCEIN ACETATE

Substrate	Activity: Fluorogenic (cell suspension)	Activity: Fluorochromatic (single cells)
Fluorescein diacetate (2.4×10^{-7} *M*)	114	67.6
3-0-Methyl-fluorescein acetate (2.1×10^{-6} *M*)	144	1.2

The concentrations of substrate were chosen so as to obtain comparable rates of hydrolysis with the cell suspensions. The fluorogenic activity is given in fluorimeter units per minute per ml of cell suspension (see Table 1 for the conversion factor). The fluorochromatic activity was determined after 30 min incubation at 23°. The figures represent the average reading of 12 cells. The individual values ranged from 17 to 165 for the cells with FDA, and from 0.7 to 1.7 for those with 3-0-methyl-fluorescein acetate. A background value of 0.8 was subtracted from the readings. The calibration factor was the same as in the experiment of Fig. 1.

The concentration of intracellular fluorescein (C_f) was found to decrease exponentially with time at rates proportional to the concentration remaining in the cells (Fig. 2) according to the equation $dCf/dt = kC_f$, where k is the rate constant of the exit process. Accordingly, this exit constant can be calculated from the slope of the curve obtained by plotting the logarithm of the concentration of intracellular fluorescein (in percentage of the original concentration) against time (Fig. 2). It was found that the cells exhibited great degree of heterogeneity with respect to the exit rate. For instance, in the experiments of Figure 2, cell *A* released half of its fluorescein in about 8 min, whereas cell *E* did it in 2 hr.

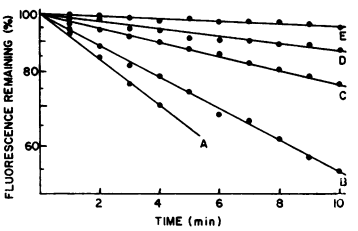


FIG. 2.—Kinetics of release of intracellular fluorescein. Each curve represents successive measurements on a single cell. See text for details on procedure and Fig. 1 for calibration factor and background. The ordinate is in logarithmic scale. It indicates the fluorescence (C_f) found in a cell at a given time expressed as percentage of the initial fluorescence (C_0). The values of C_0 ranged from 80 to 250 units.

Fluorochromasia as a property of viable cells: Experiments were designed to test whether cells of the 2B2 cultured line which gave a negative fluorochromatic reaction were nonreactive variants. Narrow glass slides were cut to fit into the mouth of 200-ml rectangular serum bottles, and an oval mark, approximately 1 × 2 cm, was etched on the slide by a diamond marker. A cell suspension was diluted to give 10^4 cells per ml. The glass slides were inserted into the serum bottles, and 0.05 ml of the cell suspension was dropped onto the oval area. An amount of medium (2 ml) sufficient to create a moist atmosphere, but not cover the slide, was added. After allowing the cells to attach to the glass slide for a period of 90 min at 37°, 13 ml of growth medium was added.

Two of the slides were kept out for "zero time" counts of fluorochromatic cells, and the other slides were removed from the bottles at 2- and 4-day intervals. The frequency of fluorochromatic cells in the oval area of the slide was recorded. The proportion of cells attached together in clones of two or more was taken as a measure of viability. It was found that the number of clones increased with time and that, in contrast to the single cells, all the cells present in the clones were fluorochromatic.

Another series of experiments were performed in order to determine whether the fluorochromatic reaction was deleterious to the cells. Cloning efficiency before and after exposure to FDA was determined. The results demonstrated that the cloning efficiency was similar in both sets.

Treatment which resulted in loss of fluorochromasia: The following experiments demonstrate that injury to the cells resulted in the loss of the ability to accumulate fluorescein. Culture bottles (3-oz) were emptied of growth medium and refilled with 0.5 ml of EDTA-trypsin. Half the cells in the bottle were released immediately by scraping with a rubber policeman. The rest of the cells were released by adding again 0.5 ml of EDTA-trypsin and incubating for about 10 min at 23°. The two resulting cell suspensions were spun down, resuspended in PBS, and assayed for fluorochromasia. It was found that among the scraped cells there were 10–30 per cent fluorochromatic-negative cells as compared with 0–9 per cent in the controls.

More drastic effects were obtained by exposing the cells to a saponin concentration of 0.5 mg per ml for 90 sec. This treatment resulted in complete loss of fluorochromasia. Likewise, freezing and thawing and aging the cells in growth medium or PBS produced complete inability to accumulate fluorescein. In all these experiments, the treatment with the deleterious agent did not cause a significant decrease in the esterase activity of the cell-free extracts.

When cells, which have been allowed to accumulate fluorescein, were damaged by puncturing with a micropipette, an "explosive" instantaneous release of fluorescein was observed. After micropuncture, the cell did not exhibit fluorochromasia. This type of experiment is illustrated in the three photomicrographs of Figure 3. Sometimes it was possible to recover inside of the micropipette a substantial fraction (estimated visually) of the intracellular fluorescein.

Fluorochromasia in other cell lines: In spite of the uniformity of the fluorochromatic reaction in the 2B2 cultured lymphoid cell line and in all other long-term cell culture lines examined in our experiments, not all cells studied gave similar morphologic appearance for positive fluorochromasia. Human and mouse erythrocytes were negative, chicken erythrocytes had positive nuclei only, protozoan cell types had fluorochromatic specific regions, and primary isolates of mammalian tissues showed wide range in the degree of fluorochromasia varying between positives and completely negative cells.

Discussion.—The hydrolysis of certain fluorescein esters of fatty acids by living cultured lymphoma cells produced fluorochromasia, i.e., an intracellular accumulation of fluorescein which could be easily visualized under the microscope.

A model depicting the fluorochromatic reaction must take account of the following experimental results: Conditions which damage the cells without destroying their morphological integrity, such as aging, treatment with surface agents, freezing, and thawing, cause lack of fluorochromasia. Mechanical injury to the cell surface by micropuncture causes an instantaneous release of the intracellular fluorescein. In the living cell, the accumulated fluorescein is excreted at a rate proportional to its internal concentration. The exit rate constant differs widely from cell to cell in a given culture. Variations of the average exit rate constant are observed under different physiological conditions.

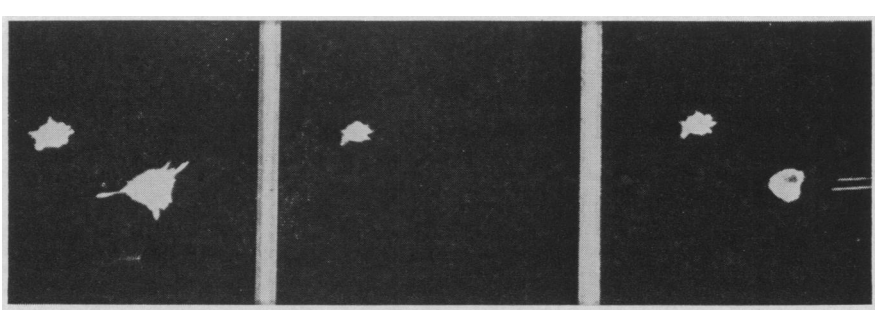


FIG. 3.—Release of intracellular fluorescein by cell damage with a micropipette. The left figure is a photomicrograph of two cells which have been allowed to hydrolyze FDA and thus have accumulated fluorescein. The two other photomicrographs were taken immediately after the cell on the right side was punctured with a micropipette. The difference between the middle and the right pictures is that for the latter the blue filter was removed from the exciting light; namely, in the first two photographs from the left, only cells with green fluorescence were visible, while in the right figure all cells are visible because they reflect white light. Polaroid film, type 410, 30-sec exposure for the two left figures and 5 sec for the third. ×160.

We propose a model which is consistent with these experimental facts. According to this model, fluorescein esters, being nonpolar compounds, penetrate readily into the cell and are hydrolyzed by esterase(s) producing fluorescein. The latter, a polar compound, cannot be extruded from the cell as fast as its esters can enter. Consequently, the rate of fluorescein production exceeds the rate of exit causing the observed intracellular accumulation of fluorescein.

A prediction derived from the proposed model is that modifications of the fluorescein molecule leading to less polar compounds as reaction products should result in increased permeability with a consequent loss of intracellular retention and lack of fluorochromasia. Accordingly, 3-0-methyl-fluorescein acetate, a fluorogenic substrate for esterases (the fluorescence yield of 3-0-methyl-fluorescein is about one third of that of fluorescein) was tested for its ability to cause fluorochromasia. Whereas this compound was hydrolyzed by the intact cells and cell-free extracts at rates comparable to those of fluorescein diacetate, no fluorochromatic activity was observed. This result agrees with the prediction, since 3-0-methyl-fluorescein is much less polar than fluorescein, especially at neutral pH.

Studies on the fluorochromatic activity of additional fluorescein derivatives gave further insight into the mechanism of the reaction. Increasing the chain length of the aliphatic acid moiety decreased the effectiveness of the ester as substrate in both intact cells and extracts. For the same acyl radical, monosubstituted esters were the most effective. This pattern of behavior probably reflects the specificity of the enzymes involved in the fluorochromatic reaction. Acetates of phenolic bases are hydrolyzed nonspecifically by many enzymes, such as proteinases, and choline esterases. Esters of longer chain fatty acids, in contrast, are hydrolyzed more specifically. For instance, the fluorochromatic activity of monodecanoyl fluorescein would involve the exclusive action of lipases, since arylesterases lack the ability to hydrolyze this substrate.

With the cultured lymphoma cell lines studied, only living cells exhibited fluorochromasia. Cloning experiments demonstrated a direct one-to-one correlation between fluorochromatic cells and cloning ability. Whereas a small proportion of single cells gave a negative reaction, all clones were positive. With cells demonstrating such a correlation, fluorochromasia is a simple and sensitive test for viable cell counts.

We have interpreted the variations of the fluorochromatic reaction in cells from primary isolates as reflections of discrete differences in cell membrane which occur during growth and differentiation, or possibly genetic heterogeneity of the population. Further studies with fluorochromatic substrates may provide information about these variations and, in addition, may be used in characterizing changes in the cell membrane after virus infection and malignant transformations.

Summary.—Lymphoid tumors maintained in culture were found to hydrolyze nonfluorescent aliphatic acid esters of fluorescein accumulating a fluorescent product (fluorescein) intracellularly. This property, *fluorochromasia,* was easily visualized under the microscope using a light source which excited fluorescein. Fluorochromasia was found to be correlated with cell viability. Cells which had accumulated fluorescent products grew into clones with the same efficiency as normal cells. All cultivated mammalian lines examined exhibited fluorochromasia. In contrast, a heterogeneity of fluorochromatic reaction was observed *in vivo.* Fluoro-

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