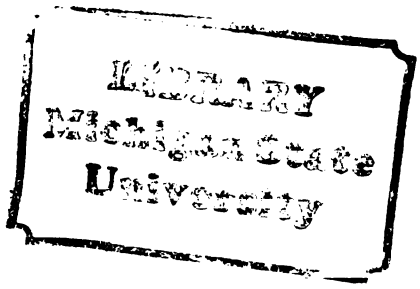

This is to certify that the

thesis entitled

DEVELOPMENT OF A GROWING DROP FLUORESCENCE DETECTOR

presented by

Claude Bruce Coffin

has been accepted towards fulfillment
of the requirements for

Masters of ~~Science~~ degree in ~~Biochemistry~~

Fritz M. Rottman
Major professor

Date June 8, 1981

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DEVELOPMENT OF A GROWING DROP FLUORESCENCE DETECTOR

By

Claude Bruce Coffin

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

DEVELOPMENT OF A GROWING DROP FLUORESCENCE DETECTOR

By

Claude Bruce Coffin

A fluorescence detector designed for use with High Performance Liquid Chromatography has been constructed. It measures the fluorescence of a growing drop, eliminating the chromatographic and spectroscopic limitations imposed by the use of a flow cell.

A chromatographic method has been developed to analyze the composition of the cap structures of messenger RNA. The method employs an enzymatic digestion of the messenger RNA, concentration on a strong anion exchange column, and subsequent separation by reverse phase chromatography. Detection is accomplished by fluorescence using the growing drop detector.

DEDICATION

This thesis is dedicated to Pam, Chris, and Robbie.

ACKNOWLEDGMENTS

The author is deeply indebted to Dr. Fritz Rottman for his guidance and personal interest throughout the course of this research. The author also wishes to express his appreciation for the help and encouragement of Drs. Jack Holland and Ron Patterson .

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LIST OF ABBREVIATIONS

RNA	Ribonucleic acid
mRNA	Messenger Ribonucleic acid
GC	Gas Chromatography
LC	Liquid Chromatography
HPLC	High Performance Liquid Chromatography
UV	Ultraviolet
PM	Photomultiplier
TTL	Transistor-transistor logic
I.D.	Inside diameter
O.D.	Outside diameter

INTRODUCTION

The transfer of genetic information from DNA to protein has long been an area of intense biochemical interest. As the general processes and mechanisms of transcription and translation have been established, much attention has focused upon the control mechanisms involved in these processes, resulting in a considerable body of knowledge concerning the regulation of gene expression.

It has become evident that gene regulation in eukaryotes occurs at both the transcriptional level, involving the turning on of the gene and the beginning of transcription, and the post-transcriptional level where the immediate gene product, a high molecular weight RNA, is processed into the much smaller, covalently modified, mature mRNA (1). These post-transcriptional processing events include excision, splicing, addition of polyadenylic acid to the 3' end of the molecule, and modification of the 5' end of the molecule to form cap structures.

The purpose of this investigation is to develop an analytical method for the efficient analysis of the composition of the 5' terminal cap structures of eukaryotic mRNA.

It was first reported in 1974 that eukaryotic mRNA, unlike prokaryotic mRNA, is methylated (2,3) but to a much lesser extent than either ribosomal or transfer RNA. Hydrolysis of eukaryotic mRNA yielded the four common nucleosides methylated at the 2' position of the ribose ring, N^6-methyladenosine, and a nucleoside later

identified as 7-methylguanosine. The results of alkaline hydrolysis indicated that most, but not all, of the methyl groups appeared in alkali resistant oligonucleotides. The general structure of these oligonucleotides was proposed by Rottman et al. (5) to be a 7-methylguanosine at the 5' terminus of the mRNA linked in a 5'-5' manner to one or two adjacent 2'-O-methylnucleosides via a pyrophosphate bond. These structures are commonly called cap structures (Figure 1). Cap structures have been found on the mRNA of many eukaryotic species and viruses that infect eukaryotes.

Properties of the cap structures

The cap compounds have some interesting attributes in common due to their unique structure. 7-methylguanosine occurs at the 5' end of almost all eukaryotic mRNA's and is the only place in the message that it is found. The methylation of guanine at the seven position introduces a positive charge on the imidazole ring (Figure 2) and when 7,9 disubstituted, as in 7-methylguanosine, lowers the pK_a of the N-1 nitrogen by two pH units (6). Furthermore, the glycosidic bond of 7-methylguanosine is less stable than that of guanosine (6). At neutral and alkaline pH's, 7-methylguanosine will ring-open between the 8 and 9 positions of the imidazole ring forming 2-amino-4-hydroxy-5-(N-methyl)formamido-6-ribosylaminopyrimidine. Finally, 7-methylguanosine has a fluorescence quantum efficiency about two orders of magnitude greater than the non-methylated molecule.

The phosphodiester linkage in the cap structure is very unusual and provides the cap structures with several interesting features.

Figure 1. The 5' terminal end of eukaryotic messenger RNA. The structures m^7GpppN are referred to as cap 0's, m^7GpppNm as cap 1's, and m^7GpppNmpNm as cap 2's.

5'-TERMINAL END

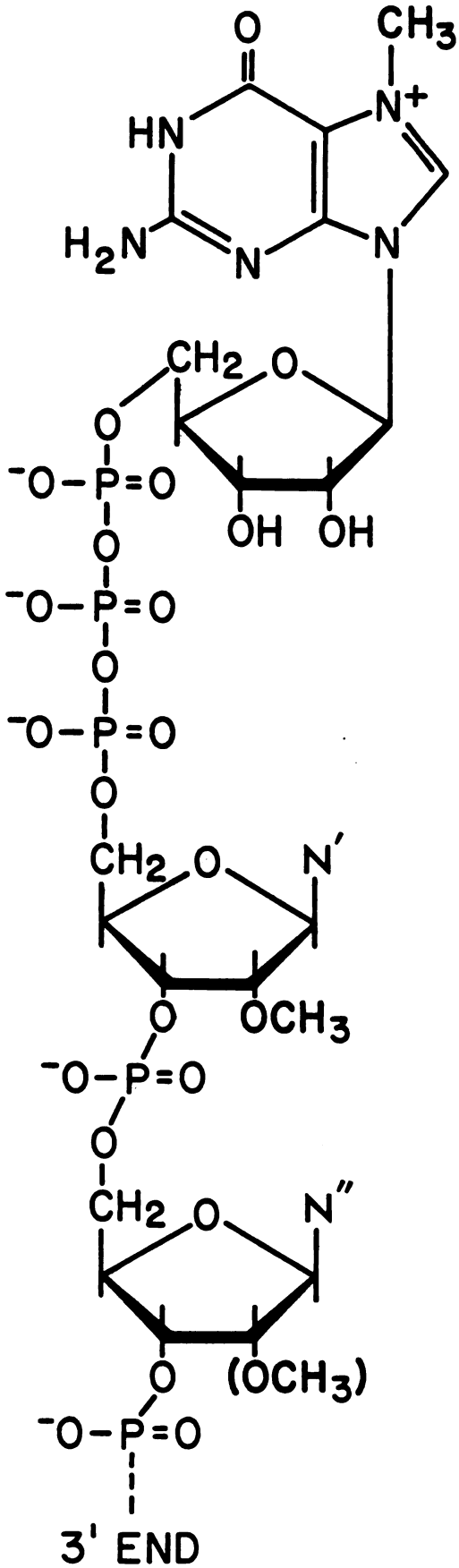


Figure 2. The 7-methylguanosine of the cap structures.

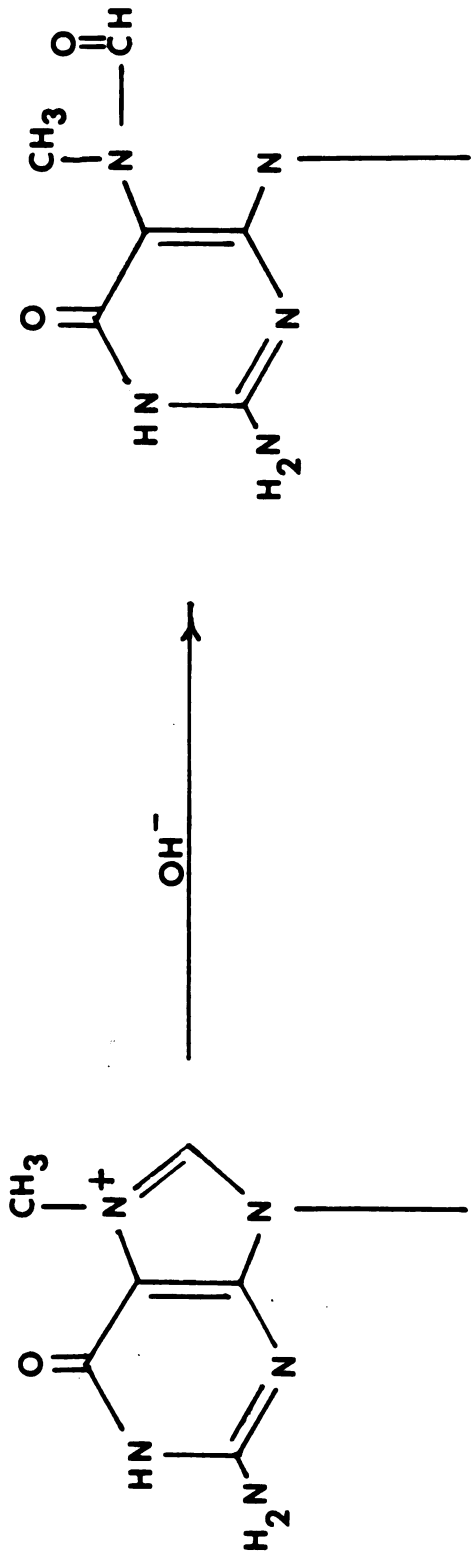


FIGURE 2

First, it links the terminal 7-methylguanosine in a 5'-5' manner to the penultimate nucleoside. The result of this 5'-5' linkage, the presence of 2',3' cis hydroxyls at each end of the mRNA, has been utilized effectively in various investigations including elimination of the cap by periodate oxidation and β-elimination (8,15,16,17), radiochemical labeling of the cap by periodate oxidation and reduction with ^{3}H-borohydride (8,9), and selective retention of both ends of the mRNA via affinity chromatography using a borate stationary phase (10,11). Biologically, the 5'-5' linkage may enhance the stability of the mRNA by preventing the action of 5' exonucleases (12). Secondly, the length of the phosphodiester bond is important to the conformation of the nucleosides in the cap structures. Studies using proton NMR suggest that, at least for the cap structure m^{7}GpppAmpA, the 7-methylguanosine can bend about the phosphodiester bond and intercalate between the two adenosine moieties (12). This unusual arrangement of the bases, providing hydrophobic and ionic regions at the end of the mRNA, requires a long, flexible spacer such as that provided by the three phosphates.

The presence of 2'-O-methyl groups in cap structures is implicated in the conformation of the cap and the possibility of base stacking (13). It causes the phosphate backbone to elongate and provide sufficient space for the terminal 7-methylguanosine to intercalate between the adjacent bases (13).

Biological role of the cap structures

The occurence of the unusual cap structures at the 5' end of nearly all eukaryotic mRNA has stimulated numerous investigations into

the possible biological role of these structures. Many of these investigations have focused upon the possible role of cap structures in protein biosynthesis.

The first report concerning the requirement for the methylation of mRNA for efficient translation described the comparison of methylated vs unmethylated viral mRNA in an in vitro, wheat germ cell-free translation system (14). It was reported that the methylated mRNA stimulated portein synthesis much more effectively than the otherwise identical unmethylated mRNA. Chemical removal of the 7-methylguanosine by periodate oxidation and β-elimination with aniline was also shown to destroy the translational ability of mRNA (8). Subsequent studies using enzymatic removal of the 7-methylguanosine (18,19) and more gentle conditions for the β-elimination of the 7-methylguanosine (15) have reinforced the findings that the cap structure is required for efficient translation of eukaryotic mRNA.

Cap analogues have also been found to affect the translational ability of capped mRNA's. 7-methylguanylic acid and similar analogues were found to inhibit the translation of a variety of capped mRNA's (20,21,22) but have little effect on the translational ability of normally uncapped mRNA (23).

In addition to the role that the cap plays in protein synthesis, the very early capping of HnRNA suggests that the cap might have important roles in mRNA synthesis and mRNA stability.

Separation methods

The development of analytical methods for the analysis of molecules extracted from biological samples is a difficult task due to the complexity of the matrix. The generally accepted approach to this problem is to selectively isolate the molecules in question. To this end, a variety of separation techniques have been developed but few have been so productively utilized as chromatography in general and liquid chromatography in particular.

Chromatography consists of a two phase system in which separation occurs as a result of a differential affinity for the phases. Gas chromatography, GC, matured earlier than liquid chromatography, LC, and separations due to very minor structural differences have been achieved by this method. The most significant limitation of GC with respect to the separation of material with a biological origin is that few biomolecules are volatile, a requirement for GC. Conversely, liquid chromatography is well suited to the separation of non-volatile, large, and ionic compounds, such as those found in a biological sample.

The first systematic development of a chromatographic separation is attributed to Tsvet (24) when he separated various biochemicals using column liquid chromatography in the early 1900's. Chromatography developed at a slow and uneven pace through the first part of the twentieth century. Although column LC was the earliest form, it developed rather slowly by comparision to gas chromatography, paper chromatography, and thin layer chromatography until the advent of high performance liquid chromatography, HPLC.

HPLC is an evolutionary technique which builds upon the theory

and successes of open column LC as well as the other chromatographic techniques. Central to the development has been the design of the column and column packing materials. These packing materials, typically based upon a silica gel support, are characterized by a small particle size (3-20 micron), a very large surface area (200-800 m^2/g), and a uniform, highly characterized stationary phase. These parameters have necessitated the development of precise, high pressure pumping systems and injection valves, and low dead volume continuous flow detectors. The one advance, however, primarily responsible for the rapid growth in HPLC is the development of bonded phase and especially bonded reverse phase packing materials. Reverse phase is a generic term encompassing a wide range of stationary phases typically consisting of straight chain alkyl hydrocarbons covalently bound to a porous silica gel (25). A wide range of selectivities are available with reverse phase material depending upon the hydrocarbon chain length, bonding chemistry, percent derivatization, pore size of the silica gel, and abundance of residual silanols (26).

Incomplete understanding of the mechanisms involved in retention on a reverse phase material has made selection of a material suitable for a given analysis difficult. Fortunately, modification of the mobile phase (27) and addition of various modifiers, e.g. ion pairing reagents (28), will almost always offer sufficient selectivity to effect the separation.

Chromatography of nucleic acids

The first reported use of column LC for the separation of nucleic acids was by Cohn (29,30) over thirty years ago using Dowex anion

exchange resins. A decade later, Anderson reported the first use of HPLC for the analysis of nucleic acids (31,32), again using Dowex resins. Limitations of the physical properties, particularly compressibility, of the gel materials led to the development of solid core ion exchange pellicular materials (33) in the late 1960's and porous silica gel materials in the early 1970's (34). These latter materials have become standard for the analysis of nucleic acids (35). The use of ion exchange chromatography is not without difficulties however and is subject to problems with stability and reproducibility.

Recently, reverse phase chromatography has been applied to the analysis of nucleic acids (9,36-42). The wide range of available stationary phases and the selectivity available through alteration and modification of the mobile phase makes possible analysis of compounds as widely divergent as positively charged bases (40) and highly negatively charged cap structures (42).

Fluorescence detection

The application of fluorescence detection to HPLC has proven particularly successful for the trace analysis of many naturally fluorescing (40) and derivatized compounds (43,45). The desirability of HPLC fluorescence detection results from the inherent sensitivity (often two orders of magnitude more sensitive than UV absorbance detection) and selectivity of the phenomena (44).

In general, all systems designed for the measurement of fluorescence contain certain components. These include : a) a UV light source, b) a device to select the excitation wavelength, c) a sample to be monitered, d) a device to select the emission wavelength, e) a

photo-sensitive device to measure the fluoresced light, and f) electronics to convert the data into a form convenient to the user. In addition, application of a fluorometer to analytical HPLC detection introduces the constraints that the illuminated sample must have a small volume and the response time of the measuring system must be rapid. Commercially available analytical fluorescence detectors meet the above requirements in a variety of ways. They all have one componenet in common, however, a flow cell. The effluent from the column is typically directed to a quartz flow cell with a volume of 10 μl to 100 μl. The design of the flow cell, especially with respect to flow characteristics and clearance times, and material of construction are critical to the performance of the detector. Significant improvements have been made by Martin et al. (50) in the design of a free falling drop fluorescence detector in which a drop falls through a light beam and also by Diebold et al. (46) in the development of a suspended drop laser fluorometer.

An HPLC fluorescence detector has been designed that employs a dripper needle suspending a growing drop. The use of a growing drop rather than a drop falling through a beam allows a much longer time for the drop to be illuminated. This enhances the signal to noise ratio and results in a very low dead volume. In this study, the sensitivity and selectivity of HPLC with fluorescence detection is utilized to develop a method to analyze the cap structures of eukaryotic mRNA.

METHODS AND MATERIALS

Detector design and construction

Optical System

The optical system of the growing drop detector is represented schematically in Figure 3. The light source consisted of either a low pressure mercury-argon lamp (Oriel Corporation, Marlboro, Mass., model # C-13-62) and power supply (Oriel model # C-73-16) or a high pressure mercury miniature arc lamp (Oriel model # 6281) and power supply (Oriel model # 6240-3). The light source is positioned either three centimeters in front of the interference filter (low pressure mercury lamp) or two meters in front of the interference filter (high pressure mercury arc lamp). The difference in distance from lamp to filter results from the presence of a colimating lens on the high pressure arc lamp housing. The image of the lamp or arc is focused immediately below the dripper needle in both systems by a pair of UV grade fused silica lenses (Oriel models A-11-621-10 0.5 " diameter, 25 mm focal length and A-11-651-30 1.5" diameter, 100 mm focal length) (Figure 4). The excitation wavelength is selected by a 1.0" circular UV bandpass interference filter (Ditric Optics Inc., Hudson, Mass.) with a peak percent transmission of 10 % at 254.0 nm and bandwidth of 7.0 nm. The emission wavelength is selected by a 1.0" square three cavity band pass interference filter (Ditric Optics Inc.) with a peak percent transmission of 38 % at 370 nm and a bandwidth of 10.8 nm. The

Figure 3. The physical layout of the components of the growing drop fluorescence detector.

FIGURE 3

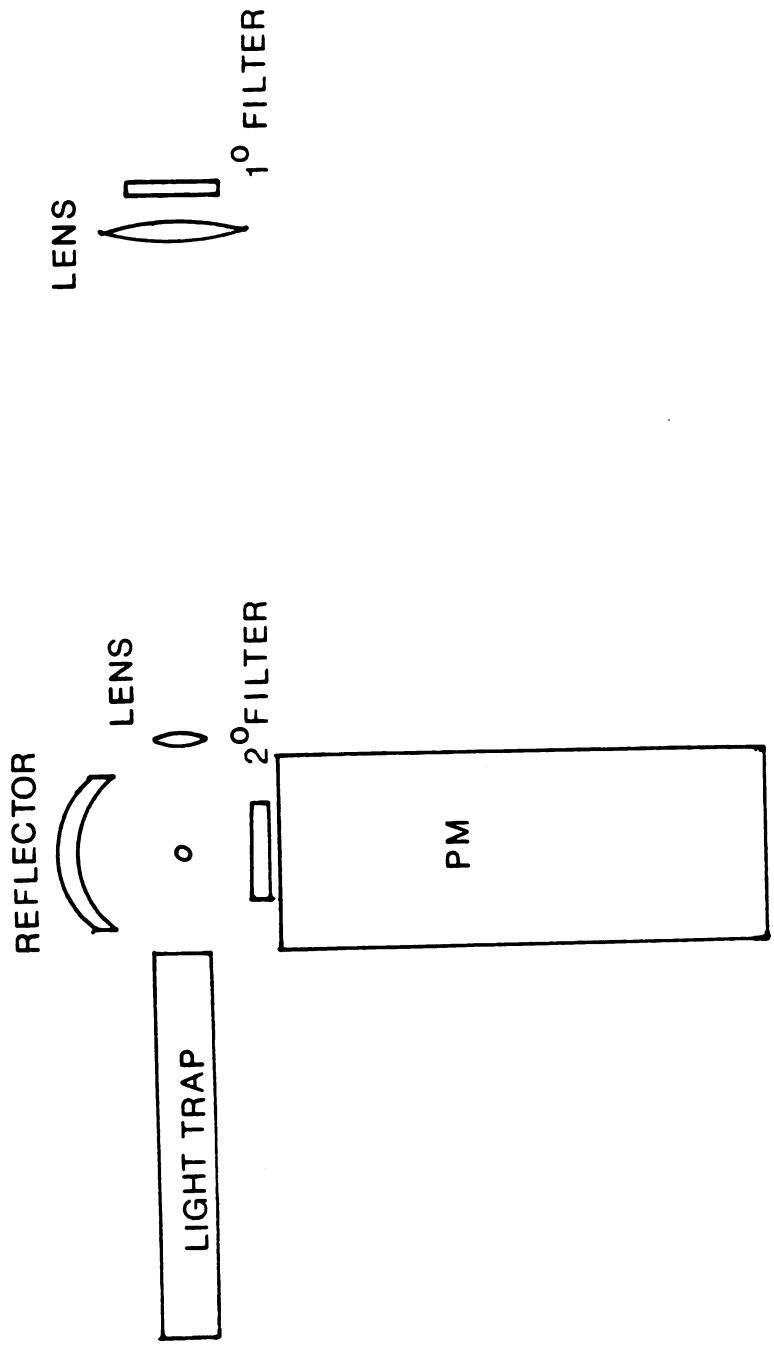
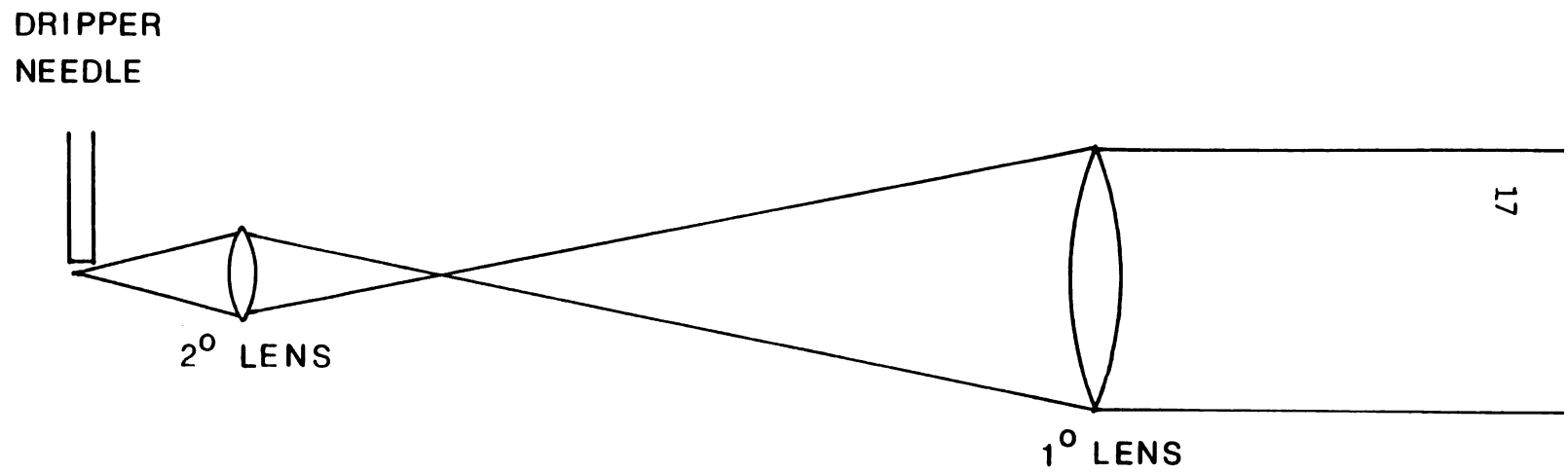


Figure 4. The optical system of the growing drop fluorescence detector.

FIGURE 4



photomultiplier tube (EMI Gencom, model 9789E) is of the end-on type with eleven stages and a 1.0 cm window. The photomultiplier is driven by a high voltage power supply (Kepco, Flushing, N.Y., model ABC) at 850-900 volts.

Liquid end

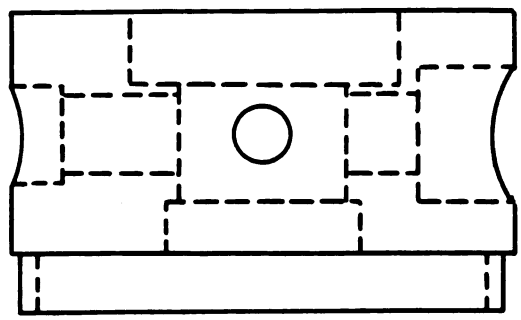
The liquid end of the detector is fabricated from a single block of aluminum and is illustrated in Figure 5. The dripper needle consists of 1/16" O.D.x 0.010" I.D. stainless steel tubing (The Anspec Company, Ann Arbor, Mi.) over which a piece of 1/8" O.D. x 1/16" I.D. teflon tubing (The Anspec Co.) has been fitted. The end of the dripper needle is immediately above the focused image of the mercury lamp and coaxial with the photomultiplier tube, see Figure 6. Opposite the photomultiplier tube is a UV enhanced parabolic reflector (Melles Griot, Irvine, Ca., model MCG 009/028) positioned such that the drop is at one focus.

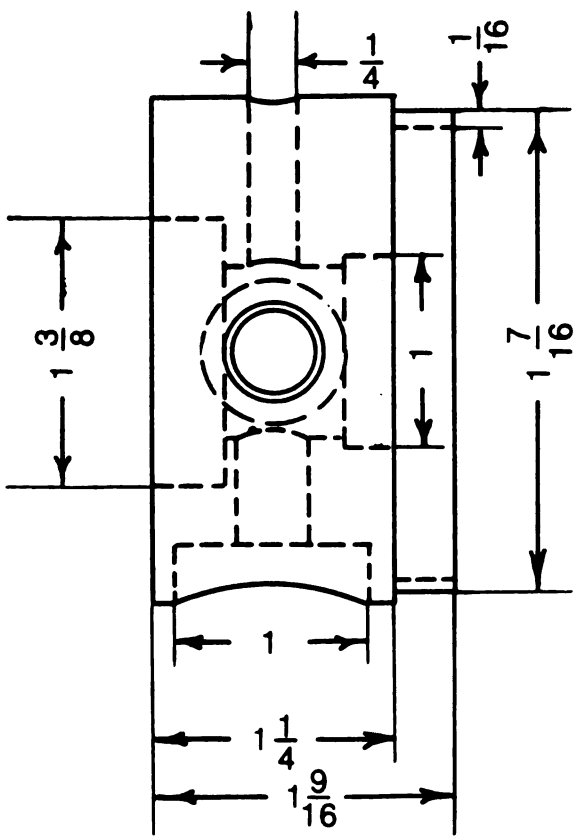
Electronics

A block diagram of the electronics and a timing diagram are presented (Figure 7) and also schematics for the amplification circuit (Figure 8) and timing circuit (Figure 9).

The operation of the detector utilizes both the fluoresced and reflected light. As the illuminated drop grows on the dripper needle, the current generated by the photomultiplier tube is amplified by a high impedance, FET input operational amplifier (Analog Devices, Norwood, Mass., model AD523J). The signal from this amplifier is filtered (Analog Devices, model AD504J) and input into a modular

Figure 5. Illustration of liquid end.




1/4
1 1/16
1 3/8
1
1 7/16
1
1 1/4
1 9/16

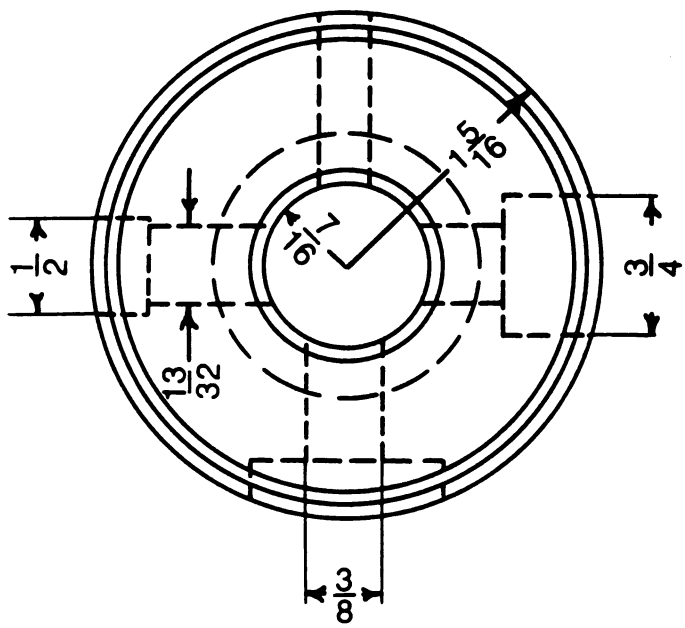

1 5/16
1 1/2
3/4
13/32
3/8

Figure 6. The dripper needle of the growing drop fluorescence detector.

FIGURE 6

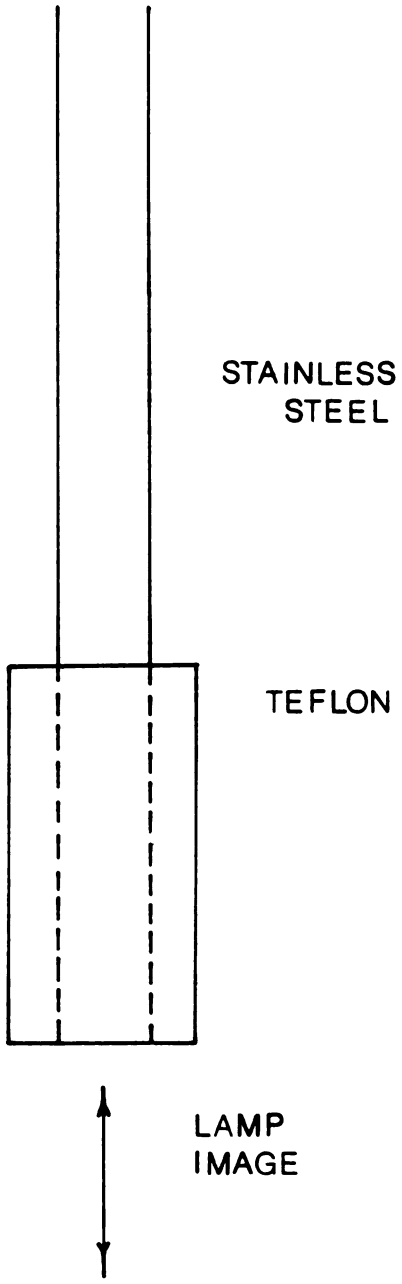


Figure 7A. Schematic of the timing circuit.

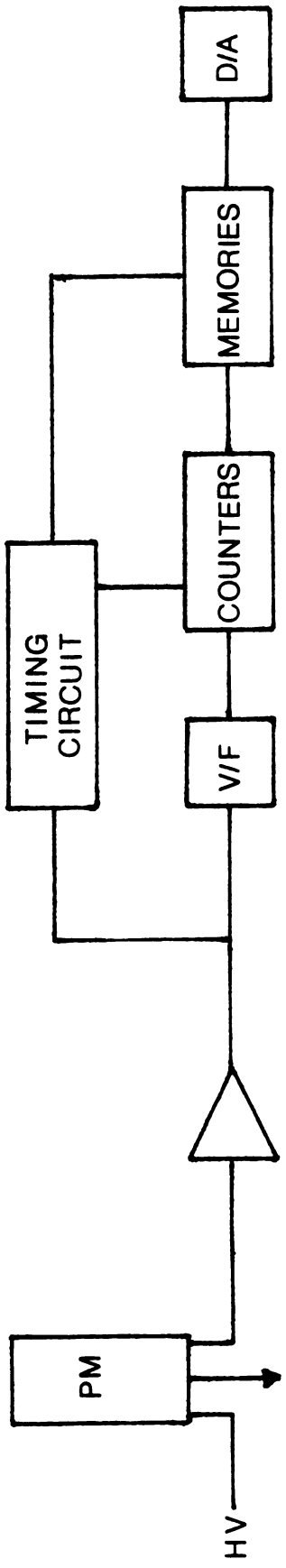


FIGURE 7A

Figure 7B. The timing sequence of the growing drop fluorescence detector.

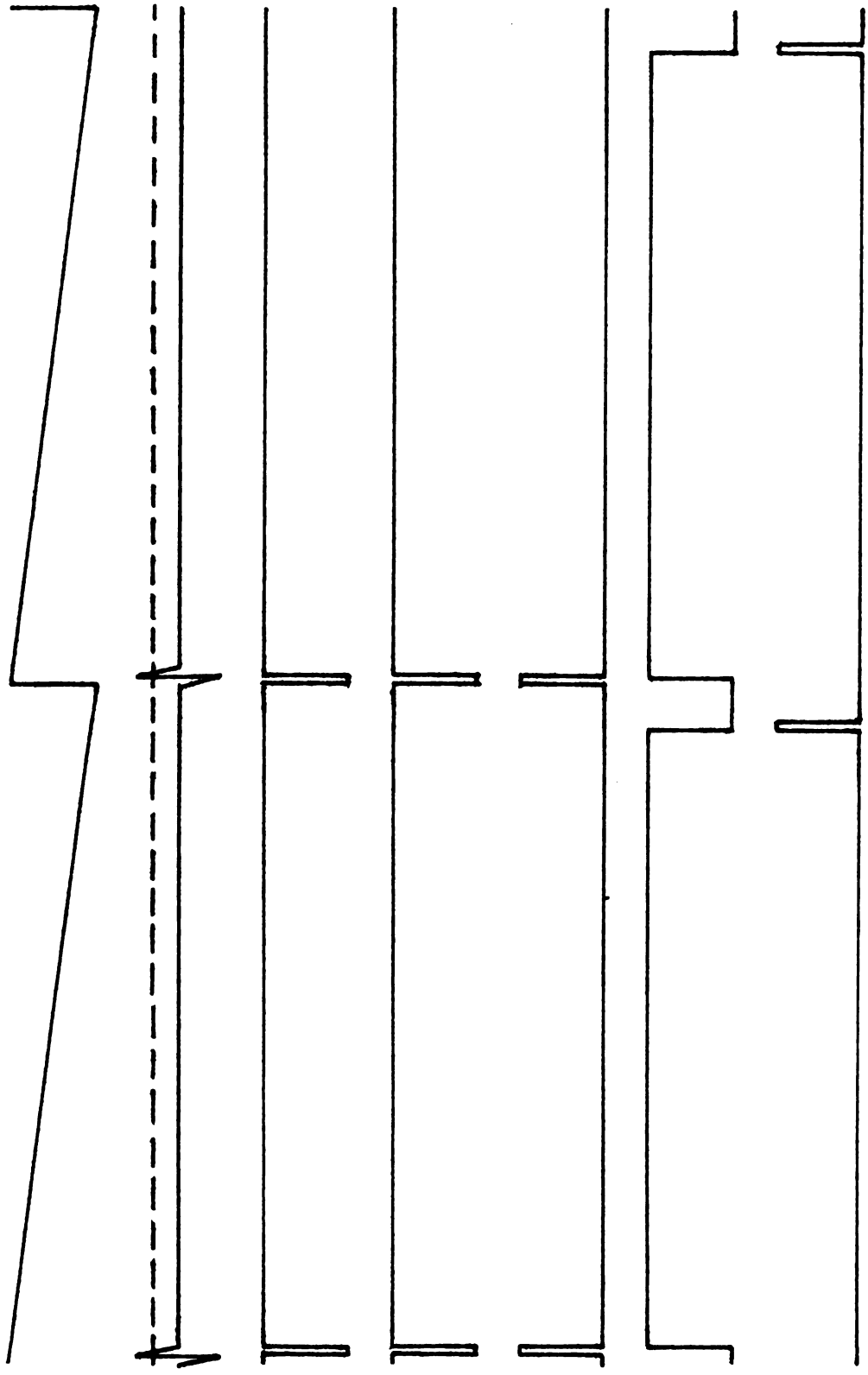


FIGURE 7B

Figure 8. Schematic of the amplification circuit.

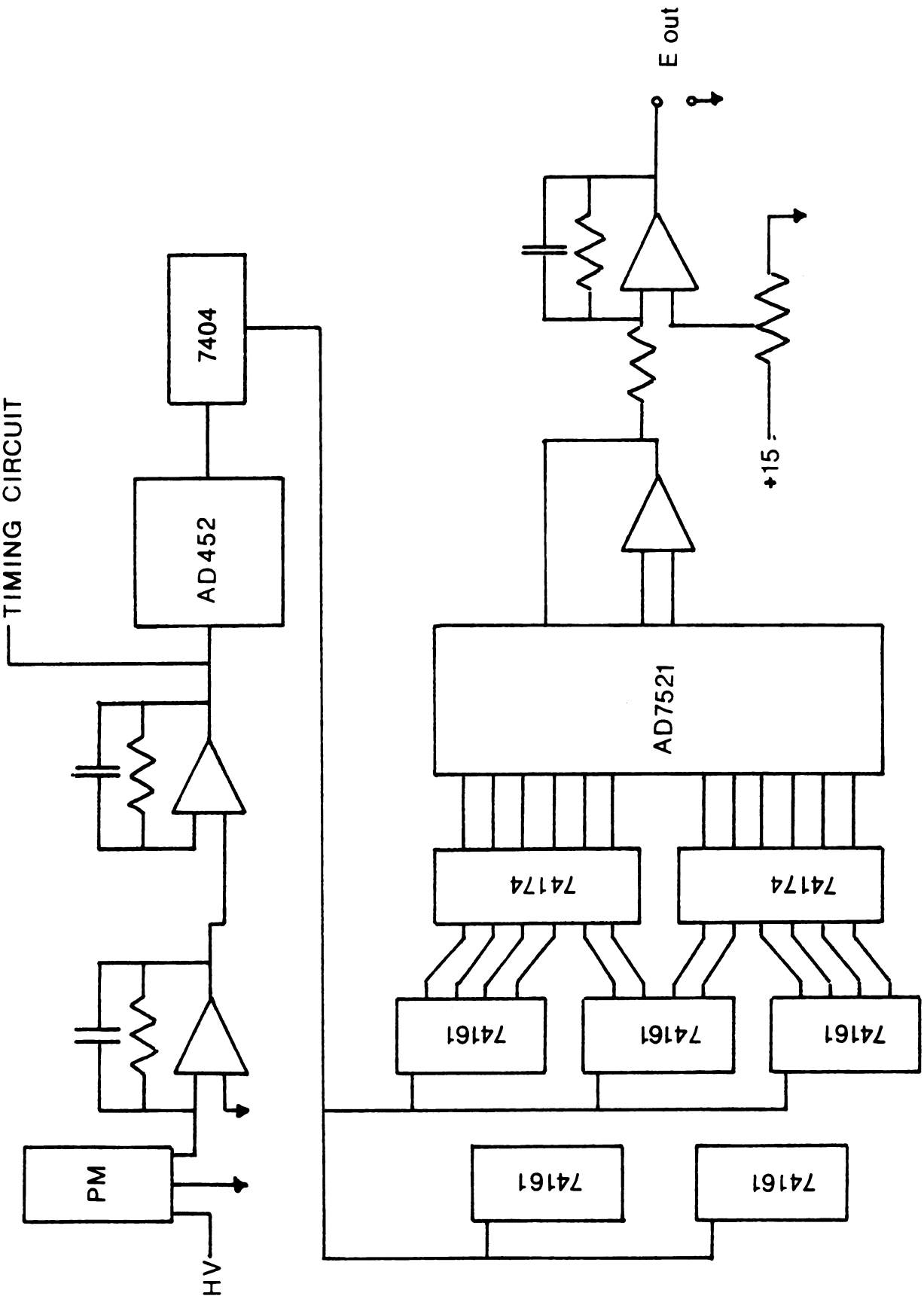

E out
+15
7404
AD452
TIMING CIRCUIT
AD7521
74174
74174
74161
74161
74161
74161
74161
PM
HV

FIGURE 8

Figure 9. Schematic of the timing circuit.

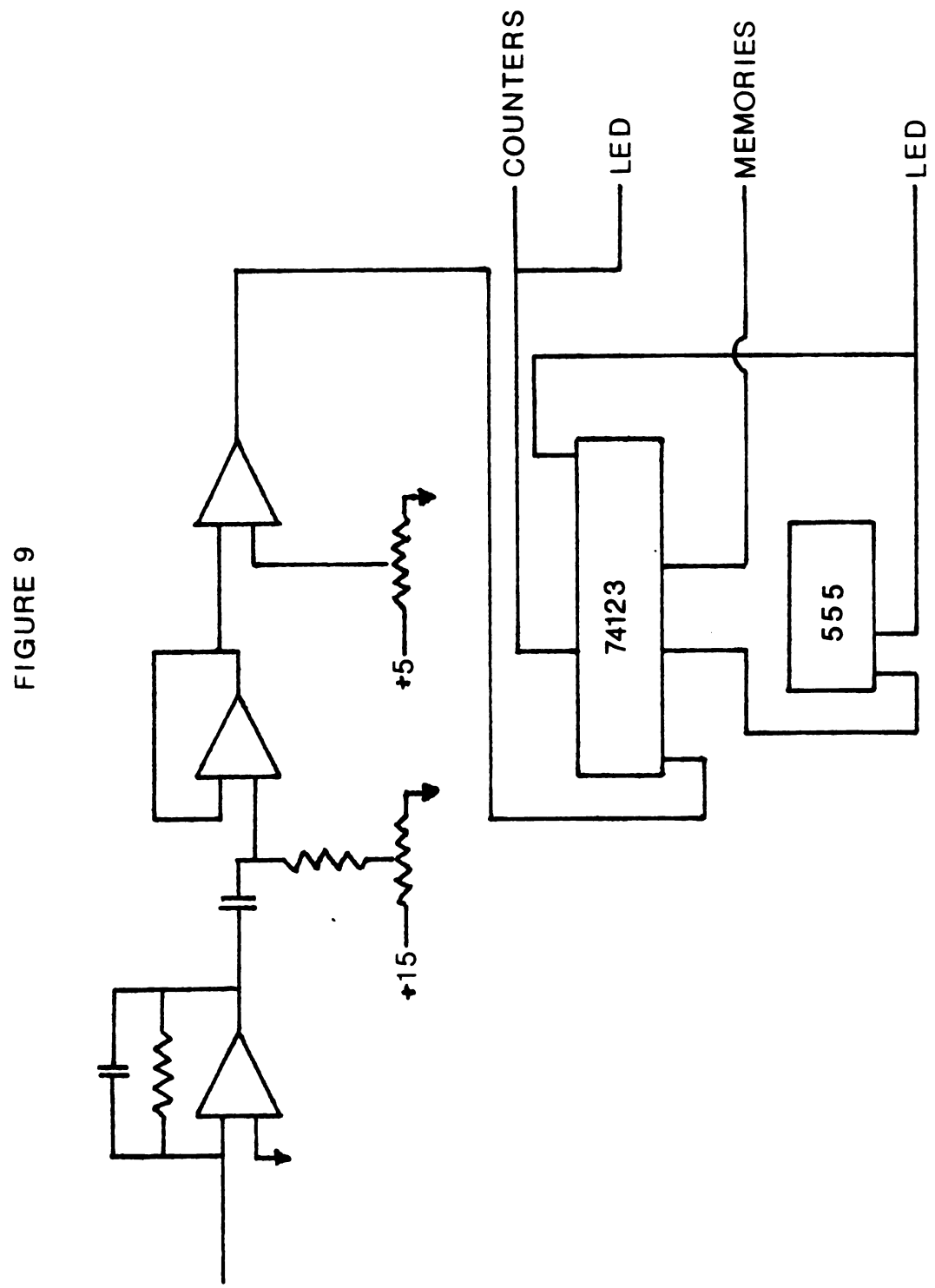


FIGURE 9

voltage to frequency convertor (Analog Devices, model AD452J) with a maximum 100 KHz frequency response. The components for the amplification and voltage to frequency conversion are mounted in the photomultiplier enclosure and immediately adjacent to the photomultiplier. The remainder of the amplification circuit is housed with the timing circuit in a separate enclosure. All components unless otherwise specified are standard 7400 series TTL. The signal from the voltage to frequency convertor is divided by two and converted to a symmetrical square wave by a flip-flop (SN7474) and used to clock a series of five 4 bit binary counters (SN74161). The output of the last three counters is input to two hex memories (SN74174). The data from these memories is input onto a 12 bit digital to analog convertor (Analog Devices, model 7521JN). The output of the digital to analog convertor is offset by biasing an output amplifier (Motorola, model 741 CP) so that the signal ranges between 0-10 mV.

The timing circuit obtains the buffered signal from the photomultiplier and differentiates it (Analog Devices, model AD504JH) then compares this signal to a reference potential. As the drop is slowly growing, the amount of light passed to the photomultiplier increases resulting in an increasing current to the first amplifier. As the drop falls, the positive voltage spike from the differentiator turns the comparator on momentarily which triggers a monostable vibrator (SN74123). One output of this monostable, Q, triggers a front panel LED and resets the counters of the amplifier circuit while the complementary output, $\overline{Q}$, triggers a second monostable (XR555CP) which stays in the ON state for a period determined by a front panel potentiometer. This monostable determines the integration time. As it turns off, it

triggers a third monostable which clocks the memories so that they latch the binary encoded output of the counters.

High performance liquid chromatograph

Chromatograph

The HPLC represented schematically in Figure 10 consisted of a Milton Roy miniPump (Laboratory Data Control, Riviera Beach, Fla., model 396-31), 0-5000 psig pressure guage (Ashcroft), Valco sample injection valve (Valco Instruments, Houston, Tx., model CV-6-UHPa-N60) with a Brownlee guard column holder and anion exchange cartridge (The Anspec Co.) in place of the sample loop, an ultraviolet absorbance detector (Laboratory Data Control, model 1203, 254 nm) and a strip chart recorder (Linear Instrument Co., Irvine, Ca., model 255).

Column

The packing material used during this investigation was 10 micron Whatman ODS-3 (Whatman Inc., Clifton, N.J.). This material was purchased in bulk and packed downward using a 10 % slurry of ODS-3 in 25 % glycerol: 75 % methanol. The packing material was sonicated for one minute then quickly introduced into the slurry reservoir and packed into the analytical column at 10,000 psig using an HPLC column packing apparatus (The Anspec Co.) utilizing a Haskell pneumatically amplified air driven pump. The eluting solvent was HPLC grade methanol (Burdick and Jackson, Muskegon, Mi.). The eluting solvent was pumped through the column for at least one hour at 10,000 psig and the bed consolidated several times. The column blank was made from 316 Li-Chroma stainless steel (Handy and Harmon), 0.25 inch O.D. by 2.1 mm

Figure 10. Schematic of the high performance liquid chromatograph and the arrangement of detectors used.

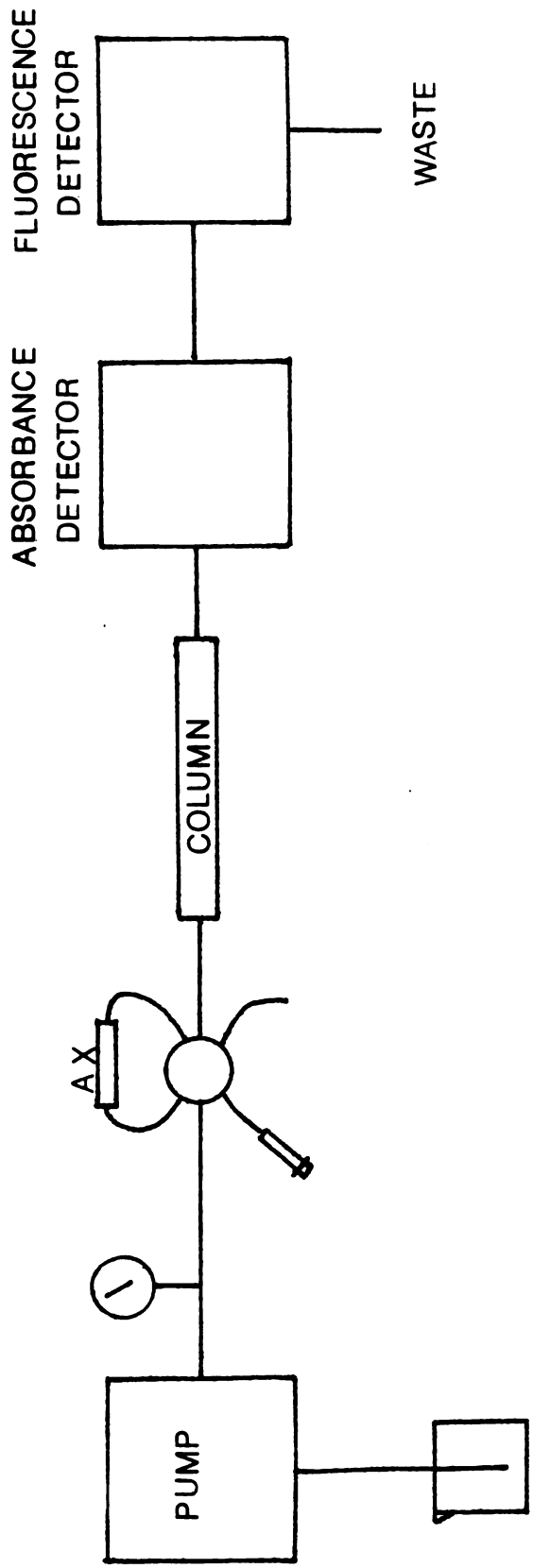

FLUORESCENCE DETECTOR
WASTE
ABSORBANCE DETECTOR
COLUMN
AX
PUMP

FIGURE 10

I.D. by 25 cm, cleaned thoroughly and fitted with Valco column end fittings (Valco Instruments). Frits consisting of a 0.125 inch diameter fritted stainless steel pressed into a 0.25 inch diameter Kel-F disc (The Anspec Co.) were used as bed supports.

Mobile phases

All solvents used were HPLC grade. The water used was double distilled (second distillation in glass) and filtered through a 0.45 micron filter (Millipore Filter Corp., Bedford, Ma., type HA). Phosphate buffer was made from analytical reagent grade phosphoric acid (Mallinckrodt, St. Louis, Mo.) and potassium hydroxide (Mallinckrodt) and filtered in the same manner as the water.

Digestion of RNA

The digestion was carried out according to the procedure outlined by Albers et al. (42). Ten A_{260} units of poly A(-) RNA were digested with 20 units of T2 RNase (Calbiochem) and 20 μg of P1 nuclease (Yama Shovu Co.) in 400 1 of 10 mM sodium acetate, pH 5, for three hours at 37°C. The pH was adjusted to 9 with 10 mM ammonium hydroxide and 5 units of dialyzed bacterial alkaline phosphatase (Worthington Biochemical Corp.) was added and the reaction mixture incubated for fortyfive minutes at 37°C. The solution was neutralized with phosphoric acid and then spiked with a mixture of cap compounds (P-L Biochemicals).

RESULTS

Linearity

The response of the growing drop detector as a function of amount of 2,5-diphenyloxazole (Research Products Inter.) injected is displayed in Figure 11. A 1 mg/ml stock solution of the 2,5-diphenyloxazole was prepared and 1:10 serial dilutions made from it. A 100 l sample loop was used and injection volumes were limited to 10 to 50 l volumes. The mobile phase was 100 % acetonitrile (Burdick and Johnson, UV) and the pump output was 0.27 ml/min. A 50 cm length of 1/16 inch O.D. x 0.30 inch I.D. teflon tubing was used in place of the column to minimize dead volume. The potential applied to the photomultiplier was either 700 or 800 volts depending on the amount of solute injected.

A linear regression analysis yielded a y-intercept of 0.61 and a correlation coefficient of 1.0.

Fluorescence as a function of flow rate

The corrected response of the detector, measured as the ratio of fluorescent signal to absorbance signal, as a function of the drop period is presented in Figure 12. These data were obtained from the isocratic separation of the four cap 0 compounds (m^7GpppN where N=A,U, G,C) plus m^7GpppGm and m^7GpppCm (P-L Biochemicals). The mobile phase was 50 mM potassium phosphate, pH 5.6, 1 g/l tetrabutyl ammonium hydroxide (Aldrich and 0.4 % tetrahydrofuran (Burdick and Jackson)

Figure 11. Response as a function of the amount of 2,5-diphenyloxazole injected.

FIGURE 11

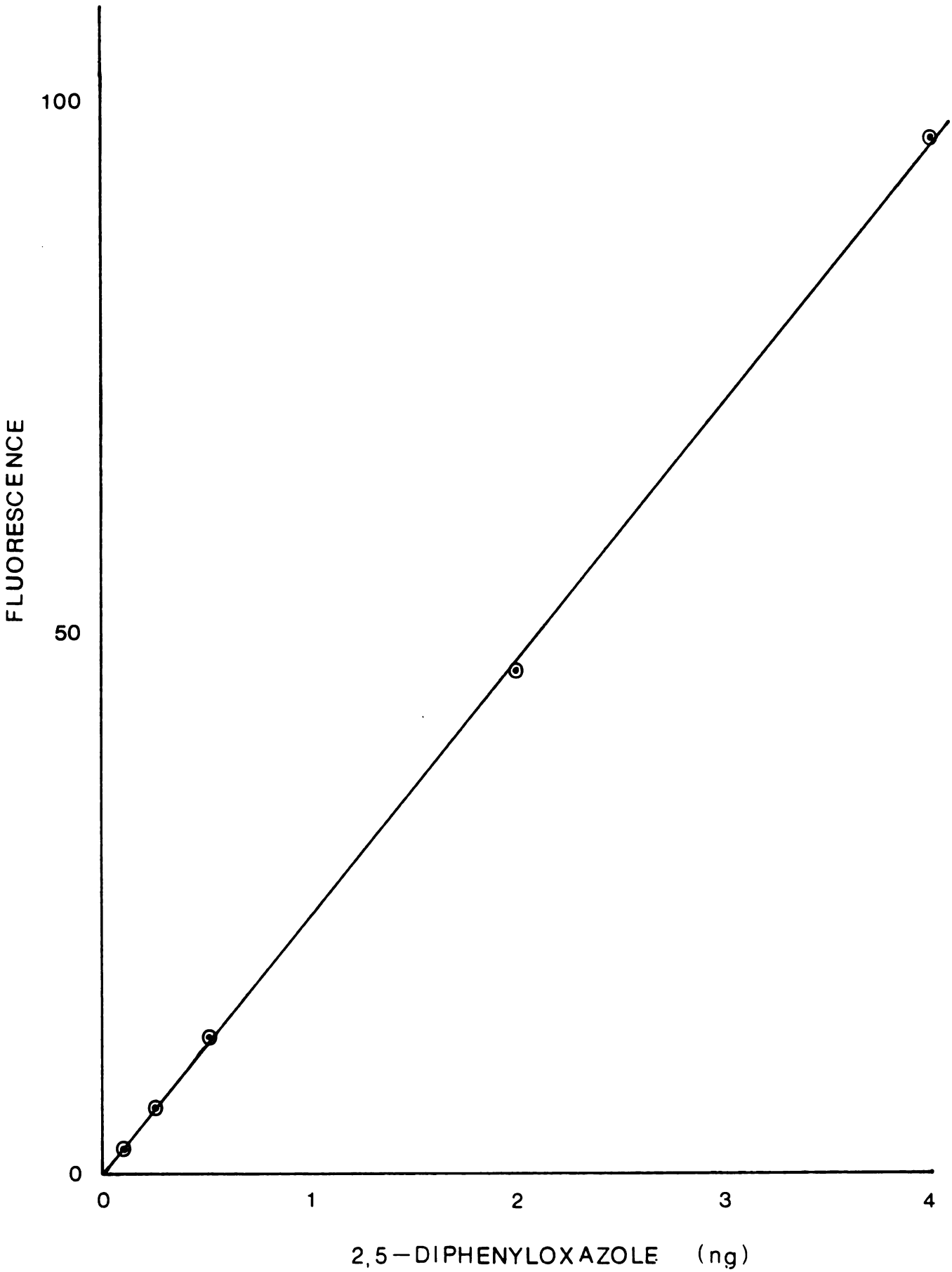
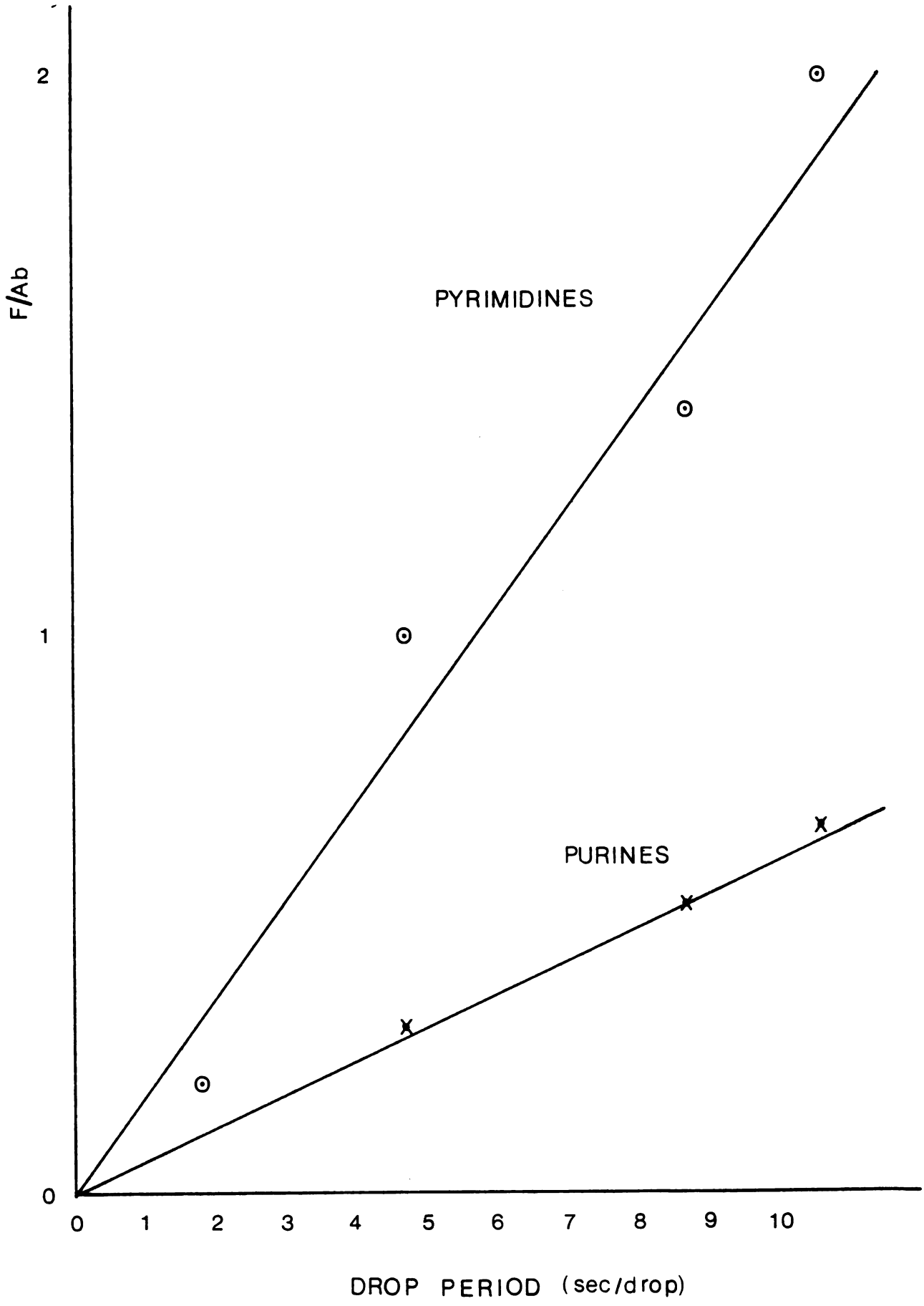


Figure 12. Corrected fluorescence as a function of flow rate. The flow rate of the HPLC was varied yielding drop periods from 1.2 seconds to 10.7 seconds per drop. The corrected fluorescence was considerably different when the penultimate nucleoside was a purine compared to a pyramidine.

FIGURE 12


F/Ab
2
1
0
PYRIMIDINES
PURINES
0 1 2 3 4 5 6 7 8 9 10
DROP PERIOD (sec/drop)

and the stationary phase was Whatman ODS-3 in a 2.1 mm I.D. by 25 cm column. The absorbance detector and fluorescence detector were in series with the absorbance detector first and the potential applied to the photomultiplier was 880 V in all cases. The absorbance detector (Laboratory Data Control) was attenuated to 0.032 A_{254} full scale.

Differential response of the caps

The corrected response of the fluorescence detector as a function of the composition of the cap structures is presented in Table I. These data were obtained from the isocratic separation of the six cap structures described in the section on fluorescence as a function of flow rate.

Separation of the cap compounds

Cap standards

Figure 13 depicts chromatograms of eight cap standards using the A) absorbance and B) fluorescence detector respectively. The analytical column was equilibrated with 50 mM potassium phosphate, 1 g/l tetrabutyl ammonium hydroxide, and 0.4 % tetrahydrofuran at a flow rate of 0.3 ml/min. The anion exchange column was switched out of the mobile phase stream and flushed with 5 ml of water. A total of 0.1 A_{254} of cap standard mix was injected onto the anion exchange column and the column flushed with 5 ml of water, removing all negatively charged material. The cap mix was then injected onto the analytical column. Upon elution of m^7GpppA the mobile phase was changed to 50 mM potassium phosphate, pH 5.6, 1 g/l tetrabutyl ammonium hydroxide, and 2.4 % tetrahydrofuran.

Table I. Corrected response of fluorescence detector as a function of the composition of the cap structures.

Drop period	Average $\frac{\text{fluorescence}}{\text{absorbance}}$		Ratio $\frac{\text{average pyrimidine}}{\text{average purine}}$
4.7 sec/drop	pyrimidines	1.0	3.3
	purines	0.30	
8.7 sec/drop	pyrimidines	1.4	2.7
	purines	0.52	
9.0 sec/drop	pyrimidines	1.7	3.3
	purines	0.51	
10.6 sec/drop	pyrimidines	2.0	3.0
	purines	0.66	

Figure 13A. Chromatogram of the cap standards using absorbance detection. The mobile phase was changed from A, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 0.4% THF to B, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 2.4% THF.

FIGURE 13A

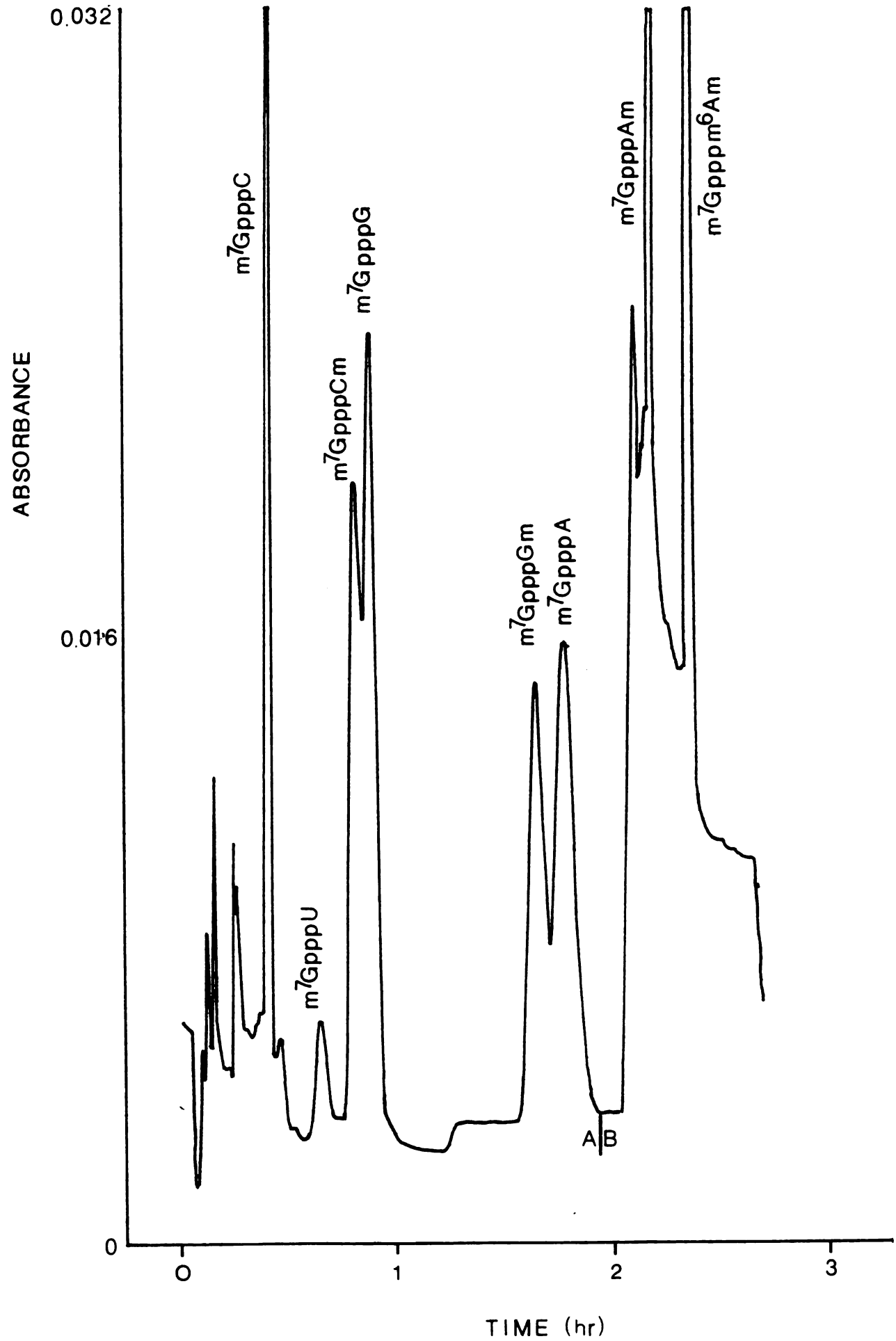
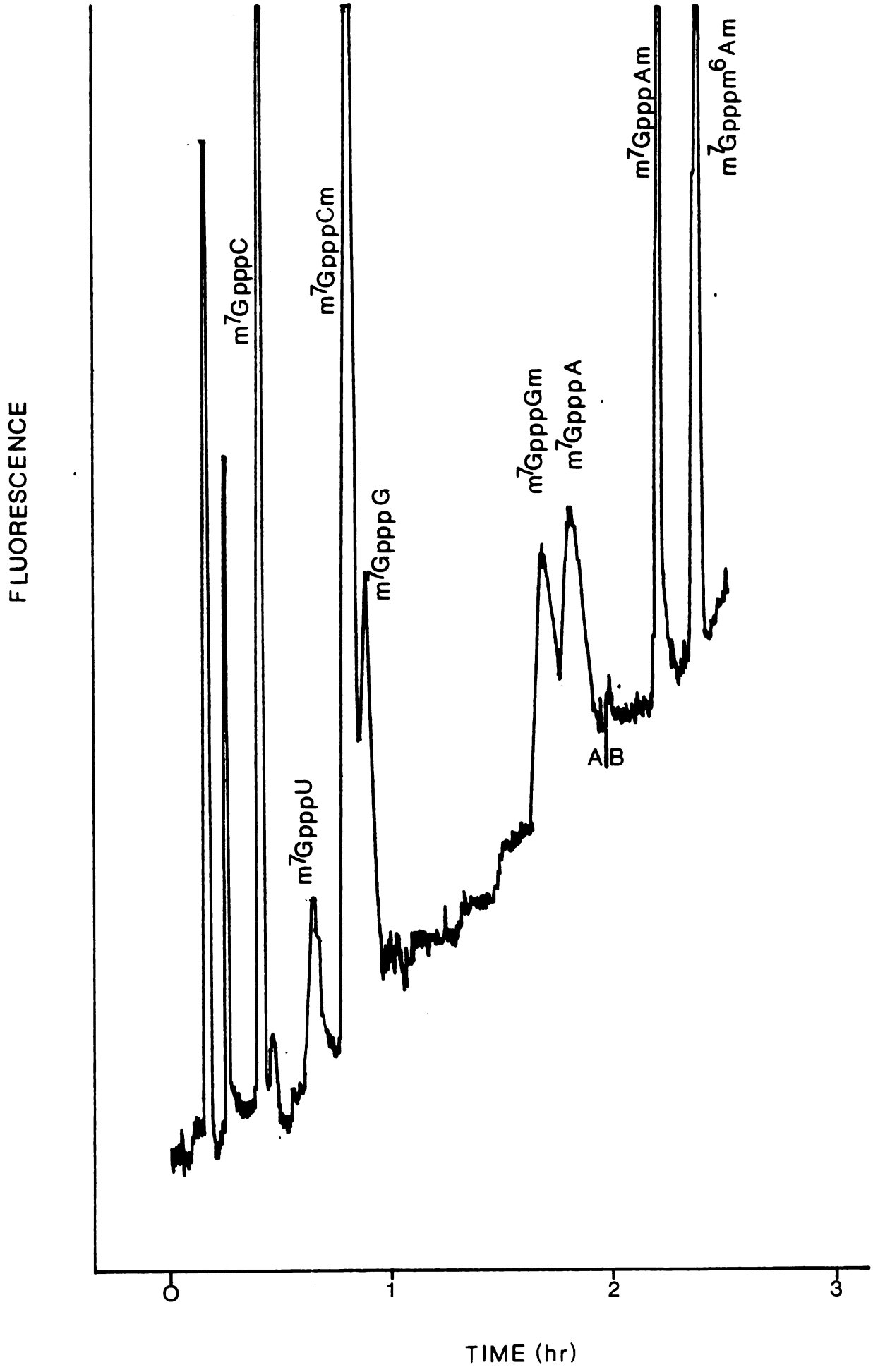

0.032
0.016
0
ABSORBANCE
m7GpppC
m7GpppU
m7GpppCm
m7GpppG
m7GpppGm
m7GpppA
m7GpppAm
m7Gppp m6Am
A
B
O
1
2
3
TIME (hr)

Figure 13B. Chromatogram of cap standards with fluorescence detection. The mobile phase was changed from A, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 0.4% THF to B, 50 mM KH_2PO_4 ph 5.6, 1.0 g/l TBA, 2.4% THF.

FIGURE 13B


FLUORESCENCE
m7GpppC
m7GpppCm
m7GpppU
m7GpppG
m7GpppGm
m7GpppA
m7GpppAm
m7Gpppm6Am
A
B
0
1
2
3
TIME (hr)

RNA blank

A chromatogram of poly A(-) RNA is presented in Figure 14. A 3.0 A_{260} sample of bovine pituitary poly A(-) RNA digested as described above was loaded onto the anion exchange column and injection onto the analytical column was accomplished as described in the previous section on the cap standards.

RNA and cap standards

Figure 15 illustrates a separation of cap standards injected along with 3.0 A_{260} of RNA. 0.1 A_{260} of cap standards plus 3.0 A_{260} of bovine pituitary poly A(-) RNA digested as described previously was chromatographed in the same manner as in the previous section.

Figure 14A. Chromatogram of poly A(-) RNA with absorbance detection. The mobile phase was changed from A, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 0.4% THF to B, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 2.4% THF.

FIGURE 14A

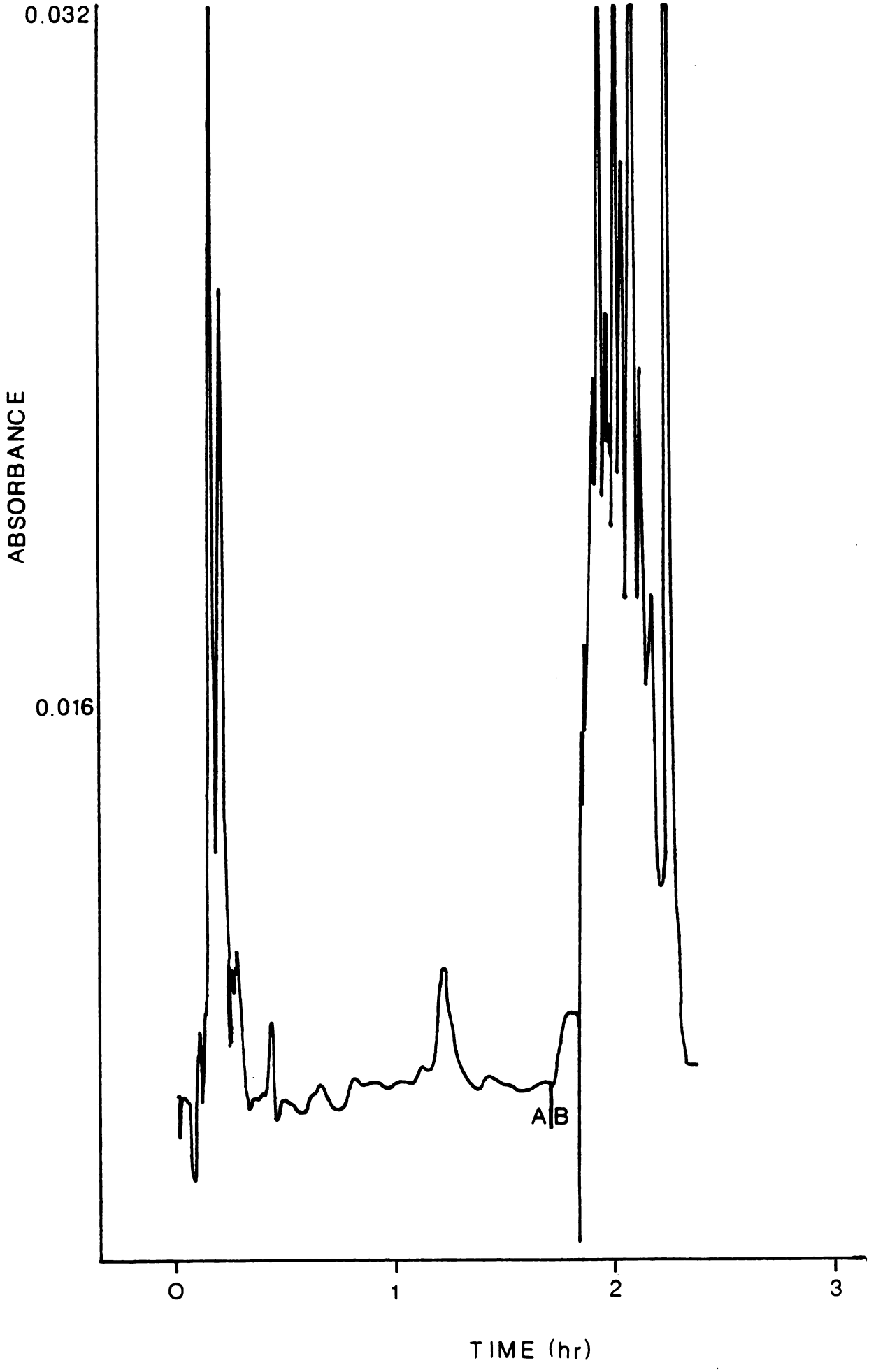


Figure 14B. Chromatogram of poly A(-) RNA with fluorescence detection. The mobile phase was changed from A, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 0.4% THF to B, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 2.4% THF.

FIGURE 14B

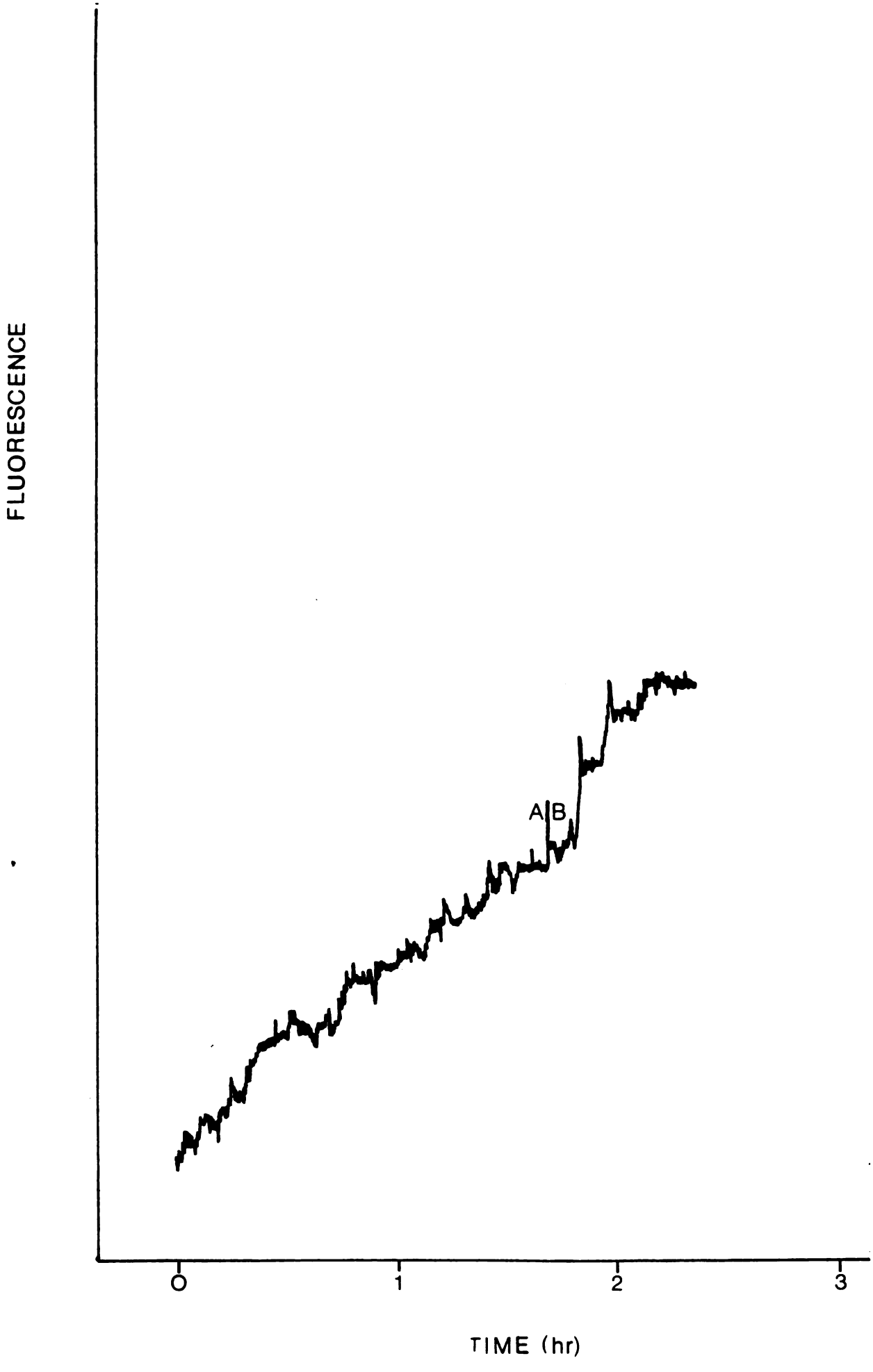


Figure 15A. Chromatogram of bovine pituitary poly A(-) RNA with addition of cap standards using absorbance detection. 3.0 A_{260} poly A(-) RNA was injected with 0.1 A_{260} of cap standards. The mobile phase was changed from A, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 0.4% THF to B, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 2.4% THF.

FIGURE 15A

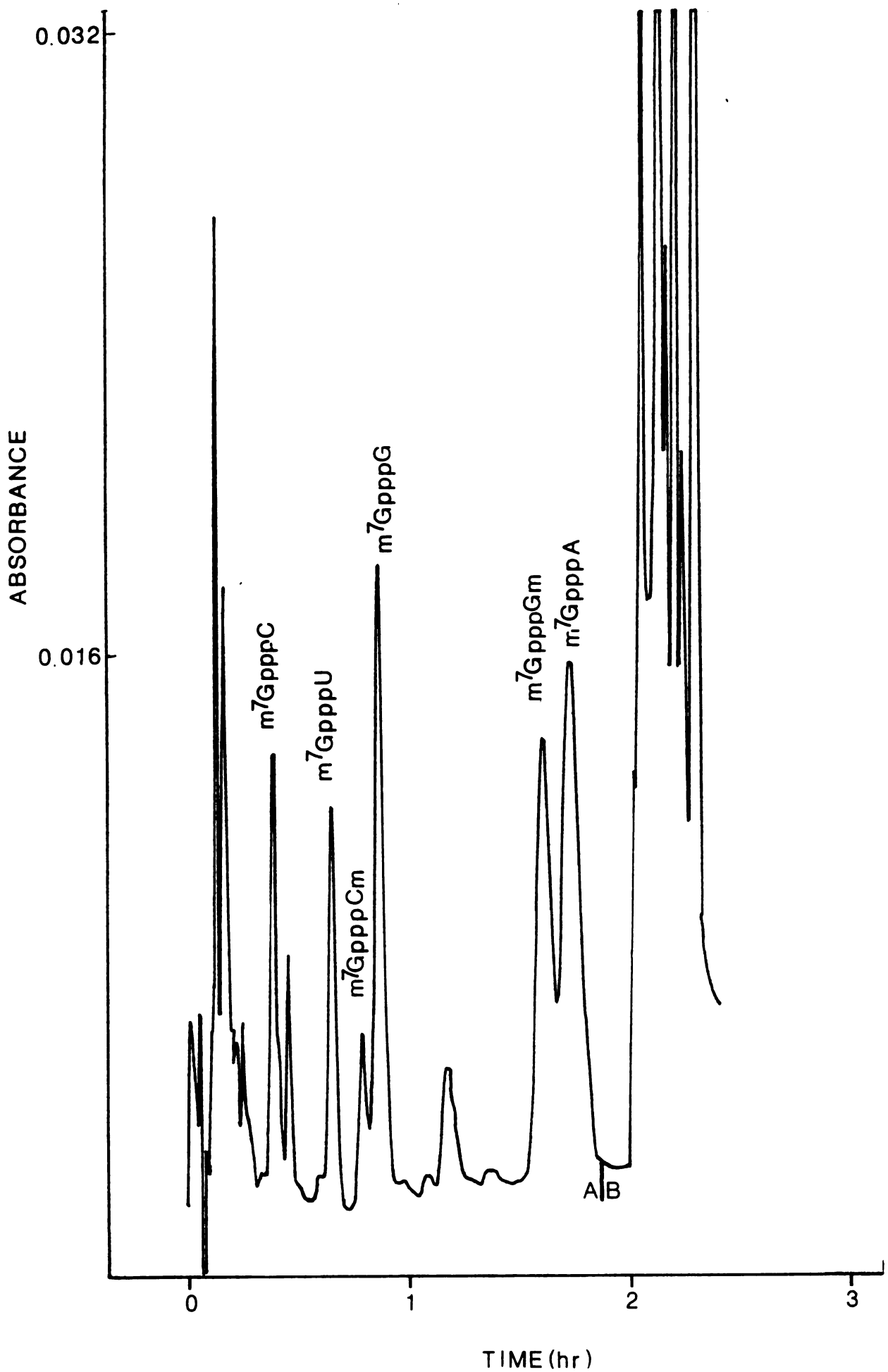
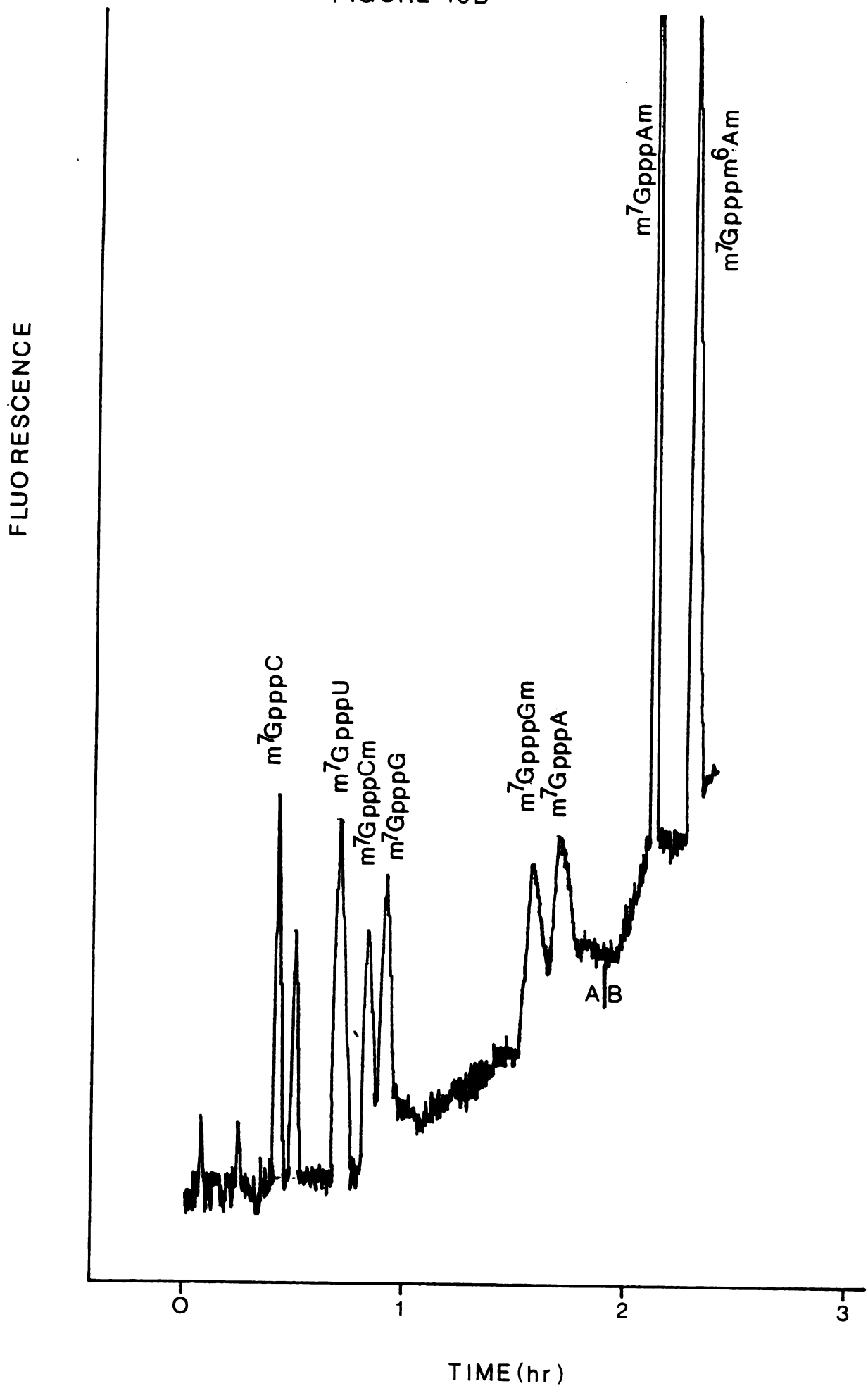

0.032
0.016
ABSORBANCE
m7GpppC
m7GpppU
m7GpppCm
m7GpppG
m7GpppGm
m7GpppA
A
B
0
1
2
3
TIME (hr)

Figure 15B. Chromatogram of bovine pituitary poly A(-) RNA with addition of cap standards using fluorescence detection. 3.0 A_{260} poly A(-) RNA was injected with 0.1 A_{260} of cap standards. The mobile phase was changed from A, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 0.4% THF to B, 50 mM KH_2PO_4 pH 5.6, 1.0 g/l TBA, 2.4% THF.

FIGURE 15B


FLUORESCENCE
m7GpppC
m7GpppU
m7GpppCm
m7GpppG
m7GpppGm
m7GpppA
A
B
m7GpppAm
m7Gpppm6Am
0
1
2
3
TIME (hr)

DISCUSSION

Detector design

Optical

The design of the growing drop fluorescence detector has been strongly influenced by the absorbance and fluorescence characteristics of 7-methylguanosine (47), the unique component of mRNA cap structures. The selection of a mercury lamp as the source of the excitation radiation was based upon its strong emission line at 253.7 nm. A 100 watt high pressure mercury arc lamp was initially selected because of its high total output and a very small arc size but was superceded by a 4.6 watt low pressure mercury-argon lamp. The low pressure lamp has a much higher output at 253.7 nm relative to 370 nm, the respective excitation and emission wavelengths of 7-methylguanosine. This change resulted in a significant reduction in the background and an increase in sensitivity. One disadvantage of the low pressure lamp is that it is not a point source but rather has an illuminated length of nearly one-half inch, resulting in a line being focused on the drop rather than a spot. The excitation wavelength is selected by a single interference filter with the maximun percent transmission at 254 nm. Multiple interference filters, optical filters, and combinations of the two were tried but invariably yielded lower signal to noise ratios.

Once light of the proper wavelength has been selected, focused on the drop, and absorbance and fluorescence have taken place, the fluoresced light must be gathered and directed to the photomultiplier. The initial design of the detector used an integrating sphere with a reflective surface to gather the fluoresced radiation. This is a satisfactory arrangement except that only a small percentage of the fluoresced light is monitered by the photomultiplier. The current design uses a relatively small cavity, a parabolic reflector, and close proximity of the photomultiplier window to the drop. This results in a significantly higher percentage of the fluoresced radiation reaching the photomultiplier.

Finally, the use of a narrow bandpass interference filter to select the emission wavelength was chosen for its high rejection of wavelengths outside the bandpass. It was found that rejection of these wavelengths resulted in a higher signal to noise ratio than did a wide bandpass absorbance filter, in spite of the loss of a great deal of fluoresced radiation with the interference filter.

Dripper needle

One of the most crucial aspects in the design of this detector is the design of the dripper needle. Various configurations have been tried including single drippers with blunt and pointed ends, dual needles with various orientations, and suspended drops using two vertically aligned needles. The configuration that proved to be the best with a variety of solvents is a piece of 1/16" O.D. stainless steel tubing with a 90° polished blunt end

over which a piece of 1/8" O.D. teflon tubing has been fitted. The teflon tubing and the stainless steel tubing must be cut at a true 90° and present a flat surface for suspension of the drop. This dripper needle design prevents creeping of the mobile phase up the side of the needle because of the hydrophobic nature of the teflon and yet allows easy positioning because of the stainless steel. Although the drop size is quite large, on the order of 50 μl, even the earliest appearing peak required fourteen drops to elute completely. Furthermore, there was no noticeable degradation in resolution or efficiency between the two detectors. This drop size might become a significant problem with more efficient or smaller columns but the problem can easily be rectified by reducing the size of both the needle and the teflon sleeve.

Electronics

Timing circuit

The timing circuit has been designed to be as simple as possible yet make the detector free from effects due to variations in the flow rate and drop residence time. The method chosen initiates the timing sequence by the falling of one drop, integrating the pulse train generated by the voltage to frequency convertor for a given period (a front panel adjustment) and then clocking the memories. Using this system, the integration has stopped and the accumulated count is stored in memory before the drop has fallen. This allows for some variation in the drop period, an interval which is more variable than the integration period established by

the timer. The detector will respond to small changes in the flow rate due to the variation in the growth rate of the drop but these effects are minor. Some care must be exercised in selecting the integration period. especially if a change in the mobile phase is made since a change in surface tension will result in a change in the drop period. If the drop period becomes shorter than the integration time, the counters will be reset before the memories have been clocked. This is easily prevented by setting the integration time based upon the mobile phase with the shortest drop period.

A number of signals have been utilized to indicated the falling of the drop including monitering the transmission or reflection of the excitation wavelength with a photocell, detecting the drop in conductance as the drop falls from two needles, and monitering the change in the signal plus background from the photomultiplier tube, the system currently in use. Although this system does limit the dynamic range of the detector, it has proven quite versatile, working with a range of solvents from water to acetonitrile, and the results are very reproducible. In order to use the background signal to trigger the timing circuit, the peak background signal must be between four and six volts. The gain must be adjusted so that a sufficient background does exist to trigger the timing circuit in the absence of fluorescence from the drop, but the gain must not be so high that the amplifiers are saturated when the strongest fluorescent peak is monitered. If this detector were to be used for more general applications, the use of a photocell that moniters the reflection of the excitation

wavelength would be the preferred method.

Amplificaiton circuit

The amplification circuit makes use of digital data transmission, integration, and storage and holds some distinct advantages over a completely analog circuit.

The original circuit design was completely analog. It used a similar current to voltage convertor and amplifier circuit but employed a sample and hold circuit to sample the potential of an analog integrator and hold that potential until the next drop fell. This circuit had significant problems with the pickup of noise on the input to the current to voltage convertor and with droop in the sample and hold circuit.

The subsequent circuit replaced the sample and hold circuit with analog to digital and digital to analog convertors. This was a substantial improvement over the previous design but the input to the current to voltage convertor was still susceptible to noise and problems with the integrator began to appear. Both of these problems are eliminated in the current design.

Presently the system is designed with the analog amplification and the voltage to frequency convertor circuits adjacent to the photomultiplier in the photomultiplier housing. This minimizes the problem of noise pickup between the photomultiplier and the first amplifier and also allows transmission of the signal from the photomultiplier in digital form which is inherently more immune to noise than analog transmission. The serially transmitted data is converted into parallel digital data by counting the pulses

from the voltage to frequency convertor. This is particularly convenient because the integration period is easily varied up to the limit of the counters. Five 4 bit counters allow for a drop period of ten seconds with the amplifiers saturated. The output from the last three counters is stored in the 2 hex memories upon the signal from the timing circuit and the digital to analog convertor outputs the updated analog signal.

Chromatography

The chromatographic separation of the cap structures is similar to the previous method used (42) with respect to the digestion of the mRNA and the fragments generated. Specifically, the cap 2 structures are degraded to cap 1 structures and the analysis consists of separating the cap 0 structures from the cap 1 structures. The composition of the cap 2 structures must be inferred from the analysis of the 2'-O-methylnucleosides. The chromatographic method utilizing the growing drop fluorescence detector is less powerful as implemented than the method of Albers et al. since it does not detect the 2'-O-methylnucleosides. It does however use a much simpler gradient system employing a one step gradient rather than a series of complex gradients. Furthermore, the method has the potential for analysing the 2'-O-methylnucleosides using conventional methods of detection.

The one feature of the current method that has allowed the great simplification in the mobile phase is the use of an anion exchange column as a selective enrichment device. This column is used in place of a sample loop and selectively retains the

strongly charged cap structures. Flushing this anion exchange column with water will selectively elute all the non-negatively charged compounds, including all nucleosides, simplifying the matrix considerably. The cap compounds are quantitatively eluted off the anion exchange column by the mobile phase designed to separate the various cap o's and cap 1's on the analytical column. The use of this enrichment column provides three important benefits. It a) simplifies the matrix sufficiently that the cap structures may be analysed without interference, b) allows the injection of large volumes without volume overloading the analytical column, and c) allows the use of microbore columns.

The analytical column used for the separation of the caps was selected after much experimentation. The column bore was selected based upon mass sensitivity and packing efficiency. Scott et al. (48,49) have established the power of microbore HPLC columns for separations requiring exceptionally high efficiency or mass sensitivity. Since the cap analysis is carried out with a very limited amount of sample, the increase in mass sensitivity with microbore columns is particularly attractive. Theoretically as the bore of the column is reduced, the mass sensitivity will increase as the ratio of the cross-sectional areas, up to the point that the column overloads. This was demonstrated empirically (data not shown) by a four fold increase in sensitivity by decreasing the column bore from 4.6 mm to 2.1 mm. The limitations to reducing the bore of the column are that extra column volumes become very significant and the smaller the bore, the harder the column is to pack efficiently. The column used in the separations presented

has a bore of 2.1 mm and is 25 cm long, the minimum length that will yield the desired separation.

The stationary phase selected for the analytical column is 10 micron Whatman ODS-3. This material was selected after an evaluation of many reverse phase packings based upon its high surface coverage, high permeability, ease of packing, selectivity, and compatability with aqueous mobile phases. The compatability with an aqueous mobile phase was a very important consideration. Although the exact nature of the surface of the stationary phase has not been adequately described, the description of a "molecular fur" is frequently used. The conformation of this "molecular fur" is a function of a variety of factors including the composition of the mobile phase. Presumably as the mobile phase changes, the conformation of the stationary phase will change to the most thermodynamically stable conformation. This is seen empirically as a change in the efficiency of the column. It was found that, of the packings evaluated, the Whatman ODS-3 was the least affected by the polar mobile phases.

The mobile phase consisted of a low ionic strength buffer, an ion pairing reagent, and an organic modifier. The separation was dependent on all three components. The phosphate buffer was chosen based upon the separation on the analytical column as well as its ability to quantitatively elute the caps from the anion exchange column. The ion pairing compound, tetrabutyl ammonium hydroxide, was in sufficient concentration that the separation was not sensitive to minor changes in its concentration. The use of tetrahydrofuran as the organic modifier was due to the

enhanced selectivity it offered with respect to $m^7GpppGm$ and m^7GpppA as compared to methanol and acetonitrile. It was found that a very low concentration of tetrahydrofuran would allow the separation of these compounds but neither methanol or acetonitrile would.

Improvements in the cap analysis

There are several improvements that could be made to this method that would enhance it. The first, and most promising, is the development of efficient microbore columns. The use of a column packing system capable of packing at 25,000 psi should allow packing efficient columns with a 1.0 mm bore. This is especially important with the fluorescence detector since the sensitivity, corrected for absorbance, is strongly dependent on the drop period and consequently the volumetric flow rate. The second improvement would be to collect the nucleosides washed off the enrichment column on a reverse phase column and separate them as was done by Albers et al. This requires the use of conventional detection systems. Finally, significant improvement can be made in the amount of sample required with the fluorescence detector by either using a post-column derivatization system to enhance the quantum efficiency of the 7-methylguanosine or use of a laser as a light source. Both methods have the potential of increasing the sensitivity at least two orders of magnitude.

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