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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

CONNIE JO KLINE

ENTITLED SYNTHESIS OF MODIFIED ECO RI RECOGNITION SEQUENCES USING T4 RNA LIGASE

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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SYNTHESIS OF MODIFIED ECO RI RECOGNITION SEQUENCES USING T4 RNA LIGASE

BY

CONNIE JO KLINE

THESIS

FOR THE

DEGREE OF BACHELOR OF SCIENCE

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Synthesis of Modified Eco RI Recognition Sequences using T4 RNA Ligase

Introduction

RNA ligase, isolated from T4-infected cells, catalyzes the ATP dependent formation of a 3'-5' phosphodiester bond between an oligonucleotide containing a 3' hydroxyl (acceptor) and an oligomer bearing a 5' phosphate (donor).[1] The RNA ligase mechanism is:[2,3]

$$E + pppA \rightleftharpoons EpA + pp_i \quad (1)$$

$$EpA + pN_np \rightleftharpoons A\text{-}5'pp5'\text{-}N_np + E \quad (2)$$

$$A\text{-}5'pp5'\text{-}N_np + M(pM)_m \overset{E}{\rightleftharpoons} M(pM)_mpN_np + pA \quad (3)$$

In step 1, RNA ligase, E, in adenylated in the presence of Mg^{++} by ATP to give adenylated ligase, EpA, and pyrophosphate, The second step consists of the formation of an adenylated donor molecule by the transfer of pA to the 5' position of pN_n, forming a 5' to 5' phosphoanhydride bond. The final step, the formation of a phosphodiester bond between donor and acceptor, occurs via nucleophilic attack of the 3' hydroxyl group of the acceptor on the 5' phosphate of the donor with the elimination of AMP. Unlike DNA ligase, RNA ligase is able to join nucleotides without the presence of a template strand. The minimum conditions are a ribenucleoside 3',5'-bisphosphate acting as the donor adding to any acceptor three or more nucleosides in length.[4,5] While the preferential substrate for RNA ligase is ribonucleotides, it is also able to catalyze the joining of deoxyribonucleotides.[6,7] These properties make RNA ligase a useful tool for synthesizing defined nucleotide sequences.

Defined deoxynucleotide sequences can be used to study the mechanisms of the Eco RI system. The Eco RI system contains a restriction endonuclease and a modification enzyme. The two enzymes recognize the same DNA sequence:

5'-G↓A*ATTC-3'
3'-CTT*AA↑G-5'

The restriction endonuclease cleaves the molecule at the two sites indicated by the two ↓ and the modification enzyme methylates the 6-amino group of the adenosine residues indicated by the *. While the two enzymes have the same recognition sequence, they recognize different functional

groups on the bases. The 2-amino group of guanosine is a recognition point for the modification enzyme but not the restriction endonuclease.[8] Replacing cytosine with glycoylated hydroxymethly cytosine results in no cleavage by the Eco RI system.[9] Introducing uracil in place of thymidine does not affect recognition by Eco RI[9], however, replacement of thymidine with 5-bromouracil decreases cleavage by a factor of ten.[10]

We plan to study the recognition sites of Eco RI on a submolecular level. Using RNA ligase, sequences of DNA will be synthesized to introduce modifications, one at a time, into the recognition sequence. The alterations will occur in both the major and minor grooves of the DNA and will affect the hydrogen bonding properties of the DNA. Normal base pairing will be conserved unless an interesting base analog has advantages that outweigh the disadvantage of altering its base pairing.

I have concentrated on studying guanosine analogs. I have successfully synthesized inosine and plan to work on another analog, such as 7-methyldeoxyguanosine, if time allows. Inosine is identical to guanosine except that it is missing the 2-amino group and in doing so eliminates one of guanosine's base pairing groups. However, the inosine-cytosine base pair still has two hydrogen baonds which is stable.

Although only six deoxynucleotides are involved in the recognition sequence of Eco RI, at least 16 nucleotides are needed to form a stable segment of double-stranded DNA. The base analogs, then, will be incorporated into octamers using RNA ligase. Binding studies will be performed to determine the V_{max} and K_m of the octamers interaction with Eco RI. These values will then be compared to those for the normal sequence, GGAATTCC. This information will give us more insight into the DNA-protein interactions occurring in the Eco RI system.

Materials and Methods

Synthesis of pdIp

Introduction I prepared pdIp by the deamination of pdAp as described by Cartright and Hutchinson.[11] The 6-amino group of adenosine is oxidized to a carbonyl group in the formation of inosine. The actual mechanism is:

$$ArNH_2 + NaNO_2 + 2HX \xrightarrow[H^+]{cold} ArN\ N^+X^- + NaX + 2H_2O$$
$$ArN_2^+ + H_2) \longrightarrow ArOH + N_2$$

Before settling on the best conditions for pdAp deamination, I tried several pilot reactions.

Pilot reactions First I tried the deamination of ATP to ITP since ATP is less expensive than pdAp. Twenty five milligrams of ATP, disodium salt, was added to 750 ul 10% acetic acid (v/v). Then, 75 milligrams of sodium nitrite was added to the mixture over two hours. The entire solution was mixed constantly in an ice bath.

The problem was to detect how much product formation had occurred. The absorbance maximum of inosine occured at 247nm and the maximum absorbance for adenosine was 257nm. This was quite a difference for nucleotides , but still did not provide for a quantitative method of determining product formation. Also, there was too much salt in the reaction mixture to make analysis by thin layer chromatography feasible. Finally, I put the reaction mixture over a DEAE-Sephadex-A25 column using triethylammonium bicarbonate buffer, pH 8.7, as described in the literature, but I could not get resolution of ITP from ATP.

I performed two more pilot reactions, one using pdAp and one using ATP again, cutting the reactants to two-fifths of the above, before I found a quantitative method to measure product formation.[12] The procedure utilized the differences in absorbance maxima of inosine and adenosine and subtracted any absorbance due to the salt. First absorbance readings at 254, 265, and 360nm were taken of the $NaNO_2$ in 3.6M sodium acetate: acetic acid buffer, pH 3.75. The 254/360 and 265/360 ratios remained the same for $NaNO_2$ throughout the reaction since $NaNO_2$ was added continually to keep its concentration constant. Then, absorbance readings at 254, 265, and 360nm were taken for each time point. The absorbance at 360nm was due entirely to $NaNO_2$ and from that the absorbances at 254 and 265nm due to $NaNO_2$ could be calculated using the ratios and subtracted from the time point readings. The corrected absorbance measurements at 254 and 265nm were then plugged into the following formulas:

$$\text{Adenosine} = \frac{1.97\ A_{265} - A_{254}}{12.8}$$

$$\text{Inosine} = \frac{A_{254} - 1.01\ A_{265}}{5.06}$$

I then did a pilot reaction using 12.1 milligrams ATP (.02 mmoles), but added it to 600 ul 3.6M NaOAc:HOAc buffer, pH 3.75.[12] Initially 27.6 milligrams $NaNO_2$ (.4mmoles) was added, but more was added throughout the reaction to keep its concentration at 0.727M. The reaction was carried out in an ice bath in the cold room with constant stirring. The results of this reaction are shown in Fig. 1.

Large scale synthesis of pdIp Using the new method of quantitation and deamination, I decided I had conditions suited for a large scale reaction. Into 550ul 3.6M NaOAc:HOAc buffer, pH 3.75, was added 0.02 mmoles pdAp and then 0.4 mmoles $NaNO_2$ was added. The concentration of $NaNO_2$ was kept at 0.727M. The entire reaction was performed in the cold room and stirred constantly. A plot of the course of the reaction is shown in Fig. 2. The reaction was stopped at 45 hours by drying the reaction mixture down and neutralizing it with TEABc buffer, pH 7.5.

Purification of pdIp The neutralized reaction mixture was applied to a 12 milliliter DEAE-Sephadex-A25 column. A 200 milliliter gradient of triethylammonium acetate buffer, pH 3.8, was used from 0.2M to 1.0M. Two milliliter fractions were collected and absorbance readings were taken at 247nm. Using the method of Shapiro and Pohl[12] I determined how much pdIp was in each fraction and how much of the total amount of pdIp each fraction represented. I pooled fractions based on these values. The elution profile of pdIp is shown in Fig. 3.

To put the pdIp in the sodium salt form, I performed a sodium precipitation of pdIp.[13] The dried down pdIp was transferred to a WA tube with four 250ul aliquots of methanol. While vortexing, ten milliliters of 1.0 M sodium iodide in acetone was added to the WA tube plus 20 milliliters of acetone. Then it was spun in a table top centrifuge and washed five times with 20 milliliter aliquots of acetone and then three times with ether aliquots. After drying in a 37°C oven for 45 minutes and overnight dessication, it was ready to be stored dry in the freezer.

Conclusions The final results of my large scale synthesis of pdIp are shown in Table 1. The values show that I achieved 12% product formation

and recovery. Since the compound is 100% pure by HPLC (Zorbax), I concluded that I did not dry the pdIp long enough and water was still bound to it , thus increasing its molecular weight.

pdIp formation by pyrophosphoryl chloride reaction of dI

Method and materials When I ran out of the pdIp made above, I decided to make more by a different method.[14] Twenty five milligrams of dI was dried overnight. The reaction consisted of adding 250ul pyrophosphoryl chloride (1mmole) to the dI. The reaction went for four hours in a -10 to -15°C salt-ice water bath. The reaction was quenched by adding ice and 0.5M TEABc buffer, pH 8.0, until its pH was basic.

Purification of pdIp The reaction mixture was purified over a 100 milliliter DEAE-Sephadex-A25 column using a two liter gradient of TEABc buffer, pH 8.0, from 0.05 to 0.5M. Ten milliliter fractions were collected and absorbance readings taken at 249nm. The elution profile is shown in Fig. 4. A sodium precipitation of the pdIp was then performed as described above and the product stored away.

Conclusions The statistics on the pdIp are in Table 2. Everything looked fine except the HPLC (Zorbax) analysis consistently gave two peaks. I could not determine what they were or separate them, so I went ahead and made more pdIp by the adenosine deamination method. Later I went back and checked this pdIp and there was only one peak given by the HPLC analysis. I put the pdIp into an RNA ligase reaction (2/2/83 - Fig. 16) and it reacted the same as the other pdIp. I concluded that this pdIp was fine to be used in reactions although I still did not know what the two peaks were or what happened to them.

Second pdIp synthesis by adenosine deamination

Since I still needed pdIp, I synthesized more by the adenosine deamination reaction described above. All conditions were identical except I used a five milliliter DEAE-Sephadex-A25 column and a 300 milliliter gradient of TEAAc buffer, pH 3.8, from 0.5 to 1.7M to purify the pdIp. The elution profile is depicted in Fig. 5. After a sodium precipitation of the pdIp as described previously, I concluded that the pdIp was synthesized correctly and pure. The final results are shown in Table 3.

Synthesis of rA_3pdG

Introduction The first step in making the standard part of the octamer was to make a oligonucleotide to ligate with pdIp. RNA ligase needs an acceptor of at least three nucleotides so I started with a segment of three riboadenosines to ligate with pdGp. I then intended to add the rest of the octamer and finally cleave off the three riboadenosines.

Pilot reactions - Conditions I performed ten RNA ligase pilot reactions before attempting a large scale reaction. The basic reaction needed acceptor, donor (excess donor for single addition reactions and equal amounts of acceptor and donor for oligonucleotide to oligonucleotide reactions), ATP, Hepes buffer (pH 7.9 for DNA-DNA joining reactions and pH 8.3 for RNA-RNA joining reactions), bovine serum albumin, dithiothreitol, Mg^{++}, Mn^{++} (for DNA-DNA joining reactions), and RNA ligase. To facilitate product analysis, I put 1ul time points in nine ul of 0.2mg/ml bacterial alkaline phosphatase so that all terminal phosphates were cleaved. Plots of the ten pilot reactions and the specific reaction conditions are shown in Figs. 6 and 7.

I noticed that the addition of Mn^{++} seemed to precipitate the donor (pdGp) and therefore give greater product formation than was actually occurring, i.e. greater than 100% product. The results only showed greater than 100% product when following the reaction by radioactivity because the donor was radioactively labeled. By precipitating the donor, the reaction was prevented from going as completely as possible. I decided to limit the Mn^{++} concentration as much as possible and to monitor the reactions some other way. I followed two reactions on the HPLC (Zorbax) and these gave reasonable results, i.e. less than or equal to 100% product.

Monitoring the reactions I began to follow the course of these reactions using radioactive pdGp labeled with 32-phosphate at the 5' position. This was prepared using 0.10 millicurie [γ-^{32}P] ATP (.025 nmoles) and 2.5 nmoles dGp. To the nucleotides I added 50 mM Tris buffer, pH 7.2 50 ug/ml BSA, 10 mM DTT, 10 mM $MgCl_2$, and 5ul PSET1 #3 (1000 units/ml). (PseT1 mutant of polynucleotide kinase) The reaction mixture was then separated on prewashed Whatman (three millimeter) paper and developed in n-propanol:NH_4OH:H_2O (55:10:35) buffer. For each ten ul pilot reaction, I used 200,000 counts per minute.

I developed time points on DE81 paper in 0.3M ammonium formate buffer. The Packard 701 B strip scanner detected radioactivity and gave a

qualitative description of rA_3pdG formation. To quantitate product formation, I cut the DE81 strips into segments and counted them in the scintillation counter.

Nearest neighbor analysis I performed a nearest neighbor analysis of the RNA ligase product of one of the pilot reactions as described Well, et al.[15] The analysis gave rA_3pdG as the only product. I decided I had conditions to perform a large scale synthesis of rA_3pdG.

Large scale synthesis of rA_3pdG From the pilot reactions I decided the reaction did not need spermine or Mn^{++} and would proceed using a relatively low RNA ligase concentration. The conditions I used were: 4 mM pdGp, 1 mM rA_3, and 2 mM ATP in 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 20 mM $MgCl_2$, 10 mM DTT, and 9uM RNA ligase in a total volume of 200ul. I let the reaction go for four hours in a 37°C water bath. I treated the entire reaction with 800 microliters 1 mg/ml BAP (final BAP concentration of 0.2 mg/ml) in a total volume of four milliliters.

Rather than using radioactivity, I followed this reaction by absorbance readings as measured using high performance liquid chromatography. I used the Zorbax column with 0.05 and 0.9M KH_2PO_4 buffer with 10% methanol (v/v). The reaction went nearly 100% after three hours.

Purification of rA_3pdG To separate the reaction mixture, I used a 12 milliliter DEAE-Sephadex-A25 column and a 150 milliliter gradient of TEAAc buffer, pH 5.8, from 0.75 to 3.0 M. I collected 1.5 milliliter fractions and read absorbances at 260nm. The elution profile is shown in Fig. 8.

Conclusions The final values on the rA_3pdG synthesis are collected in Table 4. The reaction worked very well and I claculated 96.5% product formation and recovery. I decided the product was pure and I was ready to use it as an acceptor in the next step of my octamer synthesis.

Second large scale synthesis of rA_3pdG

Materials and methods I needed more rA_3pdG to continue RNA ligase reactions and performed a large scale synthesis using the same conditions as above only in a total volume of 1000ul. To purify the product I used a 12 milliliter DEAE-Sephadex-A25 column only this time with a 200 milliliter gradient of TEABc buffer, pH 7.5, from 0.1 to 0.8 M. The elution profile is depicted in Fig. 9.

Conclusions The final statistics on the second synthesis of rA_3pdG are shown in Table 5. The reaction conditions yielded 90% product formation and recovery. This synthesis yielded enough rA_3pdG to perform all the RNA ligase reactions I needed.

Synthesis of $rA_3pdGpdI$

Pilot reactions The next step in my modified recognition sequence synthesis was to add the base analog, pdIp, to the acceptor, rA_3pdG. I performed twelve pilot reactions before settling on conditions appropriate for a large scale reaction. Courses of the pilot reactions are plotted in Figs. 10, 11, and 12. I tried changing Mg^{++}, Mn^{++}, ATP and RNA ligase concentrations, varying temperature, adding dimethylsulfonate, and increasing reaction time.

Basically, I found that the reaction needed Mg^{++} and a low concentration of Mn^{++}. If the reaction ran too long, ribonuclease activity from the RNA ligase solution started to degrade my product. Adding DMSO helped by speeding up the reaction to increase product formation before much degradation could take place. To help get rid of nucleases, I also started using autoclaved solutions, Eppendorf tubes, and pipette tips. Overall, this RNA ligase reaction took much longer than the previous joining reaction of $rA_3$and pdGp.

After treating the time points with BAP, I monitored the reactions on the HPLC. I used a Zorbax column with 0.05 and 0.9M KH_2PO_4 buffers with 10% methanol (v/v). To quantitate product formation, I determined extinction coefficients for rA_3pdG and $rA_3pdGpdI$ in the KH_2PO_4 buffer and divided these numbers into the areas calculated by the HPLC for each peak. I then determined $rA_3pdGpdI$ formation relative to rA_3pdG having a value of unity.

Large scale synthesis I used the following conditions for my large scale RNA ligase synthesis of $rA_3pdGpdI$: 4 mM pdIp, 0.5 mM rA_3pdG, and 1 mM ATP in 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 15% DMSO (v/v), and 11.40 uM RNA ligase. The total reaction volume was 350ul and I let the reaction proceed for 42 hours and five minutes in a 17°C water bath. I treated the entire reaction mixture with 700ul 1 mg/ml BAP (final BAP concentration of 0.2 mg/ml),in a total volume of 3.5 ml, for one hour and 15 minutes at 35°C.

Purification of $rA_3pdGpdI$ I purified the $rA_3pdGpdI$ using a 12 milliliter DEAE-Sephadex-A25 column and a 150 milliliter gradient of TEABc buffer, pH 7.5, from 0.2 to 1.0M. I collected 1.5 milliliter fractions and took absorbance readings at 260nm. The elution profile is shown in Fig. 13.

I was able to isolate a small amount of $rA_3pdGpdI$, but it was only 85% pure by HPLC (Zorbax) analysis. Then I used a five milliliter DEAE-Sephadex-A25 column with a 200 milliliter gradient of TEABc buffer, pH 7.5, from 0.3 to 1.0M to clean up the $rA_3pdGpdI$. I collected two

milliliter fractions and read absorbances at 260nm. Teh elution profile is depicted in Fig. 14.

The column seemed to make the $rA_3pdGpdI$ yellow, so I put the product on prewashed Whatman (three millimeter) paper). This was developed in n-propanol:NH_4OH:H_2O (55:10:35). After this, the $rA_3pdGpdI$ was finally clean although there was very little of it left.

Nucleoside analysis of $rA_3pdGpdI$ To make sure I had synthesized the correct compound, I performed a nucleoside analysis on my product.[16] The $rA_3pdGpdI$ (0.12 OD units) was incubated with 50 mM Tris buffer, pH 8.0, 5 mM $MgCl_2$, 0.1 mg/ml venom phosphodiesterase, and 0.1 mg/ml BAP C in a 37°C water bath for two hours. The reaction mixture was then analyzed on a C-8 HPLC column using 50 mM sodium phosphate buffers, pH 5.9, with and without 25% methanol (v/v). Standards of rA:dG:dI (3:1:1) were prepared to compare to the reaction mixture. The results were questionable and are tabulated in Table 6. I have currently not determined what the compound is definately, but am working on it and am certain that it will turn out to be $rA_3pdGpdI$.

Conclusions The final results on this compound are depicted in Table 6. If the product is $rA_3pdGpdI$, there was only 9.43% product formation and recovery. The results were not good enough and I did not isolate enough $rA_3pdGpdI$ to continue with my octamer synthesis, so I tried more RNA ligase pilot reactions.

Pilot reactions The pilot reactions were basically modifications of the large scale reaction and plots of these reactions are shown in Figs. 15 and 16. A compound seemed to be forming that was heavier than product that I thought might be a 3' adenylation of my product.[17] This side reaction is dependent on a high ATP concentration, so I tried lowering the ATP concentration and using an ATP regeneration system.[18] Still it was the highest ATP concentration that gave the largest product formation.

Large scale synthesis of $rA_3pdGpdIp$ For this large scale reaction, I increased the overall concentrations of pdIp, rA_3pdG, and ATP, but kept their relative proportions the same. I also added the $MnCl_2$ as the final reagent to help limit any donor precipitation by $MnCl_2$. The conditions of the large scale reaction were: 8 mM pdIp, 1 mM rA_3pdG, and 2 mM ATP in 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM DTT, 15% DMSO (v/v), 11.40 uM RNA ligase and 17°C and a total volume of 250ul. I only let this reaction go for six hours to decrease the possibility of product degradation. I did not treat this reaction

with BAP so I could isolate the pdIp after the column purification.

Purification of $rA_3pdGpdIp$ The reaction mixture was purified over a 12 milliliter DEAE-Sephadex-A25 column using a 250 milliliter gradient of TEABc buffer, pH 7.5, from 0.2 to 1.2 M. I collected two milliliter fractions and took absorbance readings at 260nm. The elution profile is plotted in Fig. 17.

Nucleoside analysis of $rA_3pdGpdIp$ The results from the DEAE-Sephadex-A25 column were confusing so I performed nucleoside analyses on the final three peaks as described above. The results are displayed in Table 7 (the second peak off of the column was not product so I discarded it) and show identical ratios for the last two peaks that look similar to the standards and identical tothe ratios of the $rA_3pdGpdI$ I synthesized before.

Second purification of $rA_3pdGpdIp$ I treated with BAP and pooled the final two peaks off of the DEAE-SEphadex-A25 column. The two migrated together on an HPLC (Zorbax) analysis, so I decided they were the same compound. The compound was only 79% pure, though, so I put the mixture over another DEAE-Sephadex-A25 column using a 200 ml gradient of TEABc buffer, pH 7.5, from 0.2 to 1.2M. I collected two milliliter fractions and read absorbances at 260nm. The elution profile is shown in Fig. 18.

Conclusions The final results on $rA_3pdGpdI$ are shown in Table 7. The nucleoside analysis is still indeterminate, so I am currently sequencing the product to provide conclusinve information. If the product is $rA_3pdGpdI$, I obtained 24.8% product formation and recovery, so this large scale synthesis of $rA_3pdGpdI$ went much better than the first one, but still was not overwhelming.

Synthesis of pdAATTCCp

Introduction The final part of my octamer was pdAATTCCp to act as a donor to add to $rA_3pdGpdI$. Bob Gould synthesized dAATTCCrC in solution by the triester synthesis method.[19] I took that compound and performed the rest of the reactions to convert it to the donor I needed.

Reactions involving dAATTCCrC The dAATTCCrC was only 82.7% pure by HPLC (C-8) analysis, so I performed a lagre scale clean-up of the compound using the C-8 column. After that it was 97.1% pure .

The next step was a periodate oxidation and beta-elimination to cleave off the terminal ribocytosine and expose a phosphate.[20] All of the dAATTCCrC (581.4 nmoles) was dried down and then 58.14 umoles of sodium periodate was added to it. This solution was incubated in the dark at room temperature for 60 minutes. Then 50ul 1.0M rhamnose was added and left to react for 30 minutes at room temperature. Finally, 2.9 milliliters of 0.33M aniline-hydrochloric acid in 0.66M sodium acetate, pH 5.0, was added to the mixture and incubated for 60 minutes at 45°C in the dark. After spinning out particulate matter and washing the precipitate with 0.3M TEABc buffer, pH 7.5, the redissolved precipitate was applied to a five milliliter DEAE-Sephadex-A25 column and eluted using a 200 milliliter gradient of TEABc buffer, pH 7.5, from 0.3 to 1.2M. Two milliliter fractions were collected and absorbance readings taken at 260nm. The elution profile is shown in Fig. 19.

The last step was to kinase a phosphate onto the 5' position to make the compound a proper donor. All of the dAATTCCp (570 nmoles) was added to 5.700 nmoles of ATP. This was dried down and 50 mM Tris buffer, pH 7.5, 50 ug/ml BSA, 10 mM $MgCl_2$, 10 mM DTT, and 100ul PSET1 #8 (Pseudomonas aeruginosa phage polynucleotide kinase) added to it in a total volume of 500ul. The reaction proceeded at 37°C for three hours and 30 minutes. I purified the reaction mixture on prewashed Whatman (three millimeters) paper developed in n-propanol: NH_4OH:H_2O (55:10:35) solvent.

After these reactions, the pdAATTCCp was only 79.0% pure, so I applied the solution to a five milliliter DEAE-Sephadex-A25 column and eluted the compound using a 200 milliliter gradient of TEABc buffer, pH 7.5, from 0.3 to 1.2M. I collected two milliliter fractions and took absorbance readings at 260nm. The elution profile is shown in Fig. 20.

Final results of pdAATTCCp The final statistics on the pdAATTCCp are displayed in Table 8. The compound is very pure and is accurate by

nucleoside anAlysis. However, the overall product formation and recovery was only 26.3% starting with the dAATTCCrC.

Conclusions

So far the synthesis of my modified Eco RI recognition sequence is going very well. The most immediate item for me to do is the sequencing of what I believe to be $rA_3pdGpdI$. When that is determined, I will have the donor, pdAATTCCp, and the acceptor, $rA_3pdGpdI$, all ready to ligate together. Then I will have to cleave off the three riboadenosines and perform sequencing reactions on my octamer. If all of that works out, I will start the kinetic studies on the octamer in comparison with the normal Eco RI recognition sequence. If time allows, I will synthesize another base analog and incorporate it into the rest of the octamer.

Overall, I learned a tremendous amount doing my Biochemistry 292 project about working in a laboratory in general and about specific lab techniques. I am very appreciative of the opportunity to gain this experience. I hope I have contributed something to the lab in return for this valuable experience.

A"P Deamination III - Pilot reaction

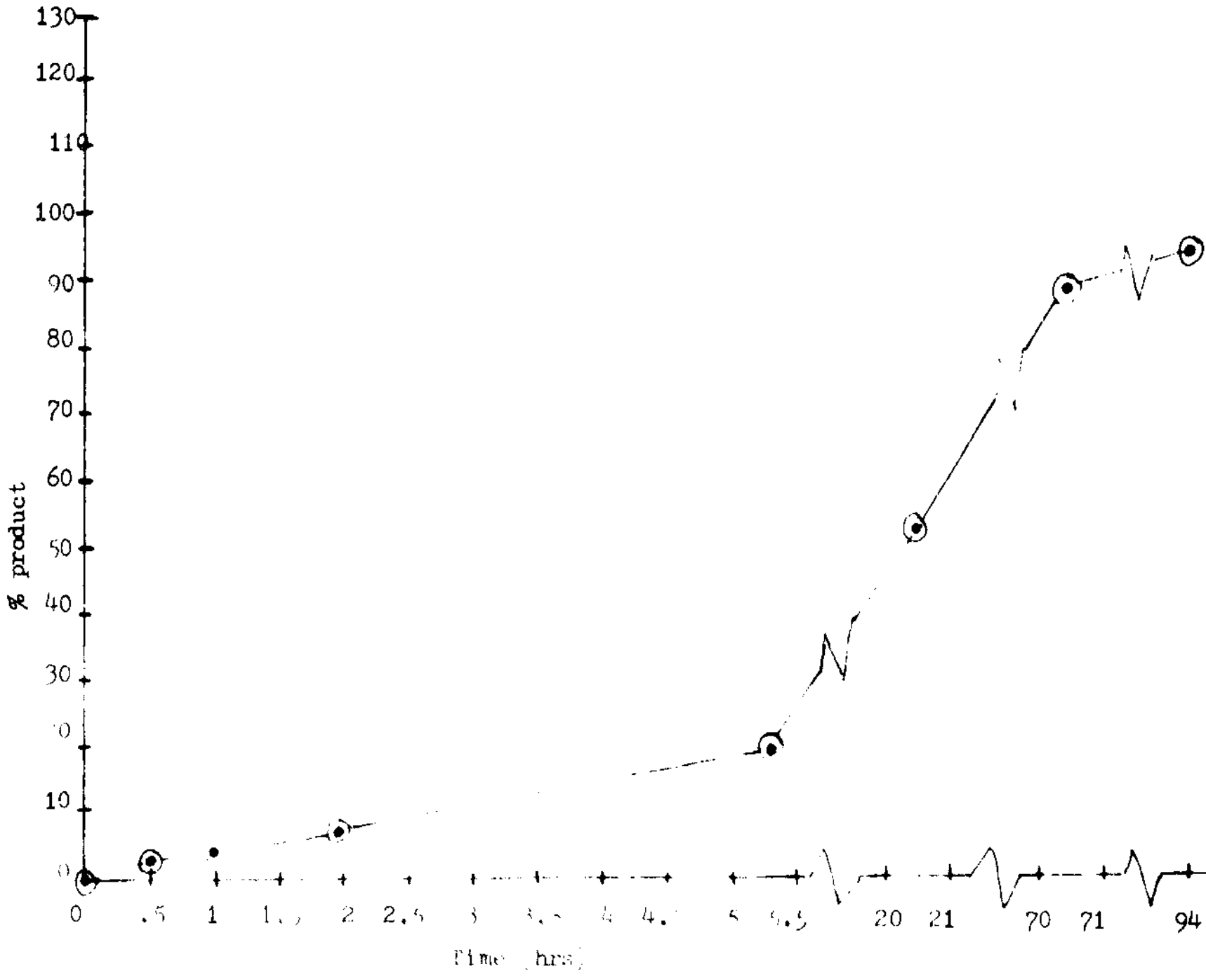


Fig. 1. Third ATP deamination reaction. 600ul 3.6M NaOAc;HOAc buffer, .0? mmoles ATP, .4 mmoles NaNO$_2$. 6/11/82.

pdAp Deamination II -Large scale

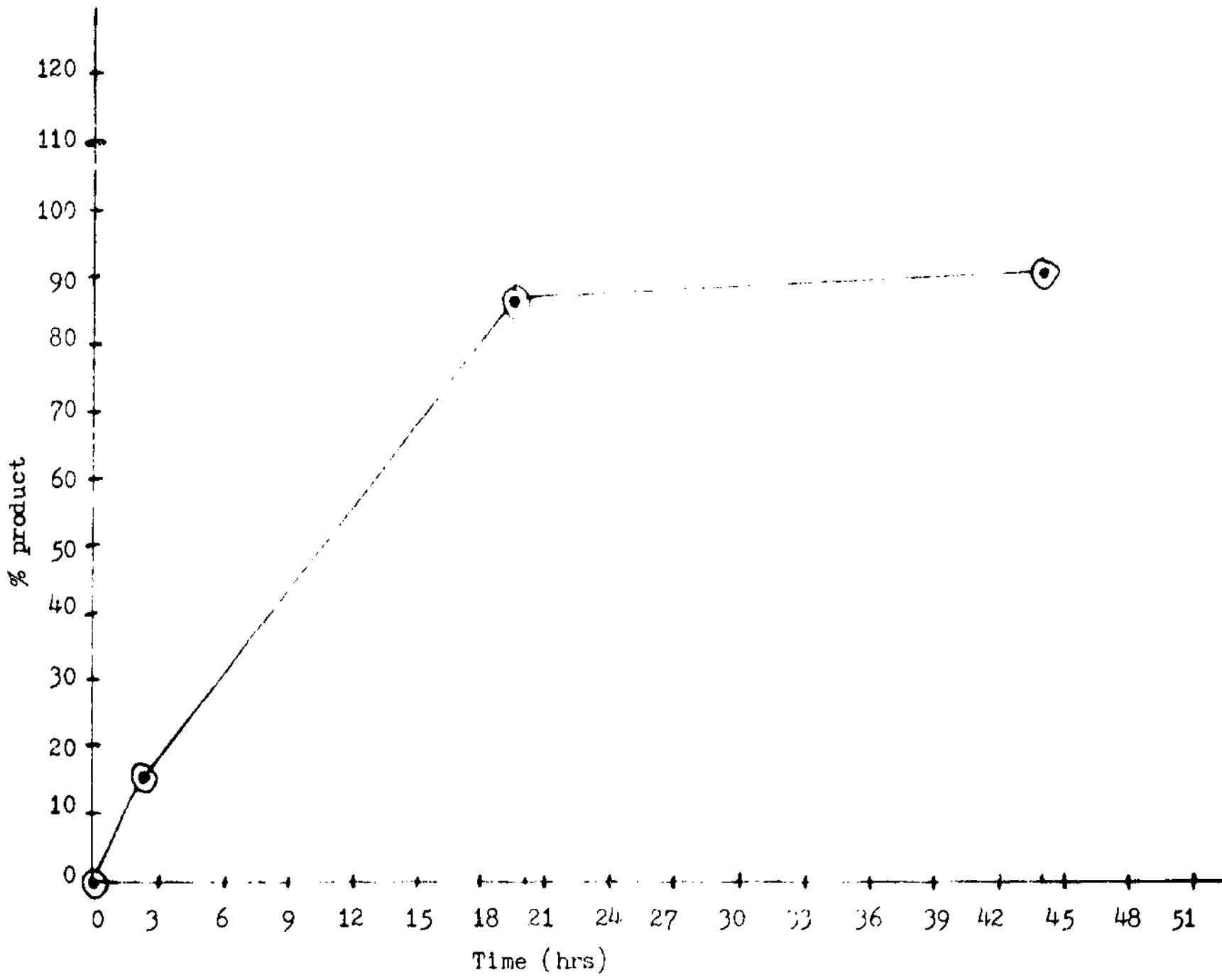


Fig. 2. Second pdAp deamination pilot reaction. 550 ul 3.6M NaOAC:HOAc buffer, .02 mmoles pdAp, .4 mmoles $NaNO_2$. 6/14/82.

DEAE-Sephadex-A25 column -
Large scale pdAp deamination reaction

Fig. 3. Elution profile of large scale reaction of 6/14/82. TEAAc buffer.

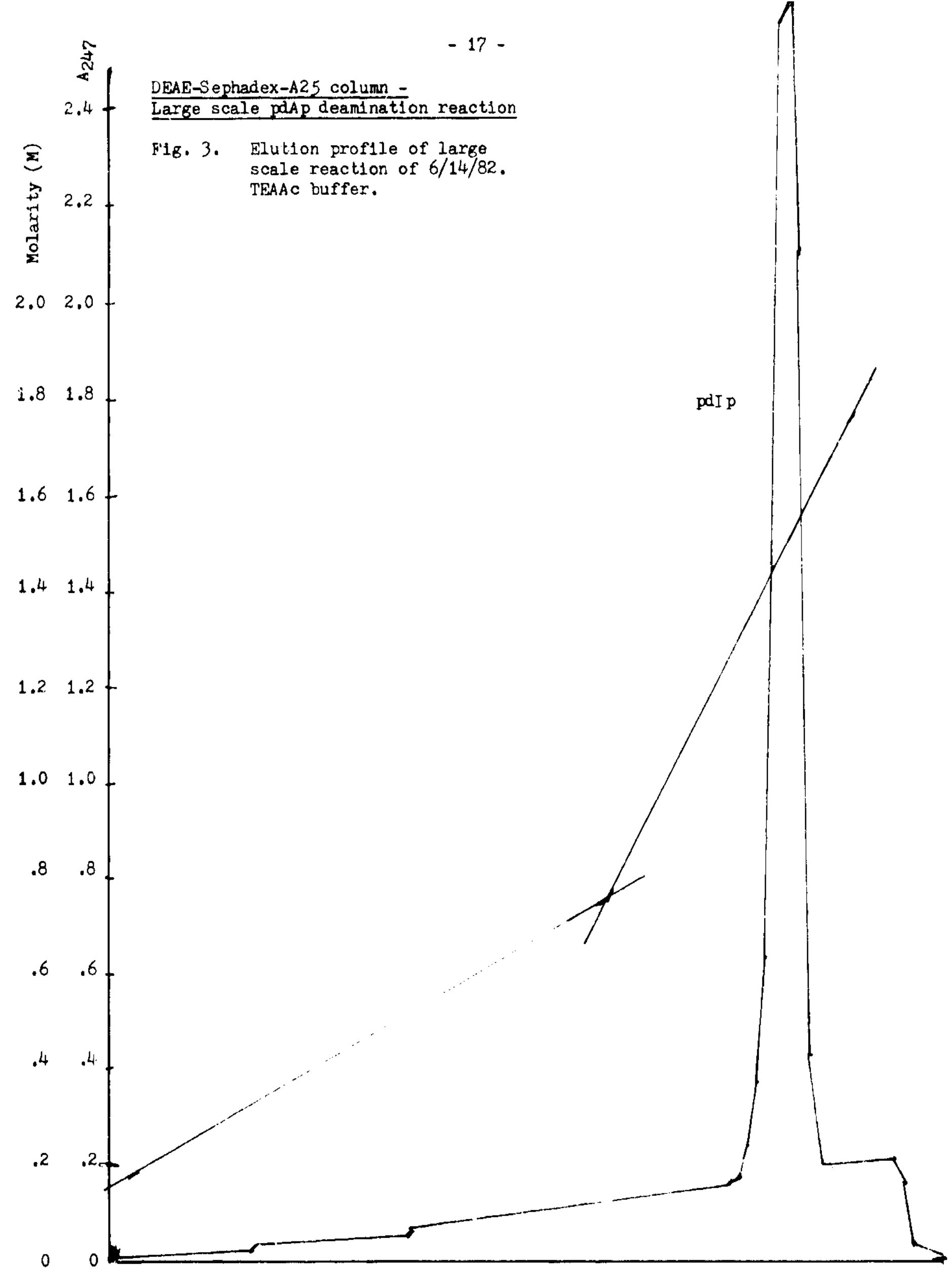


Table 1

Final results on large scale synthesis of pdIp
Deamination of pdAp

Conditions of synthesis: 3.6 M NaOAc-HOAc buffer, pH 3.75, .4 mmoles $NaNO_2$, .02 mmoles pdAp. Kept $NaNO_2$ concentration at 0.73M by adding more $NaNO_2$. Final volume - 550ul. Stopped reaction at 45 hours. 6/14/82.

	250/260	280/260	290/260
Actual ratios	1.58	0.32	0.14
Calculated ratios	1.68	0.25	0.03

Absorbance peak at 248 nm.

Purity on the HPLC - 100%

Total amount of pdIp synthesized - 0.025 mmoles
(I only started with 0.02 mmoles pdAp and since the pdIp seems to be pure, I think some water was left in my product after dessication.)

DEAE-Sephadex-A25 column - Phosphoryl chloride reaction of dI

Fig. 4. Elution profile of phosphoryl chloride reaction of 11/6/82. TEABc buffer.

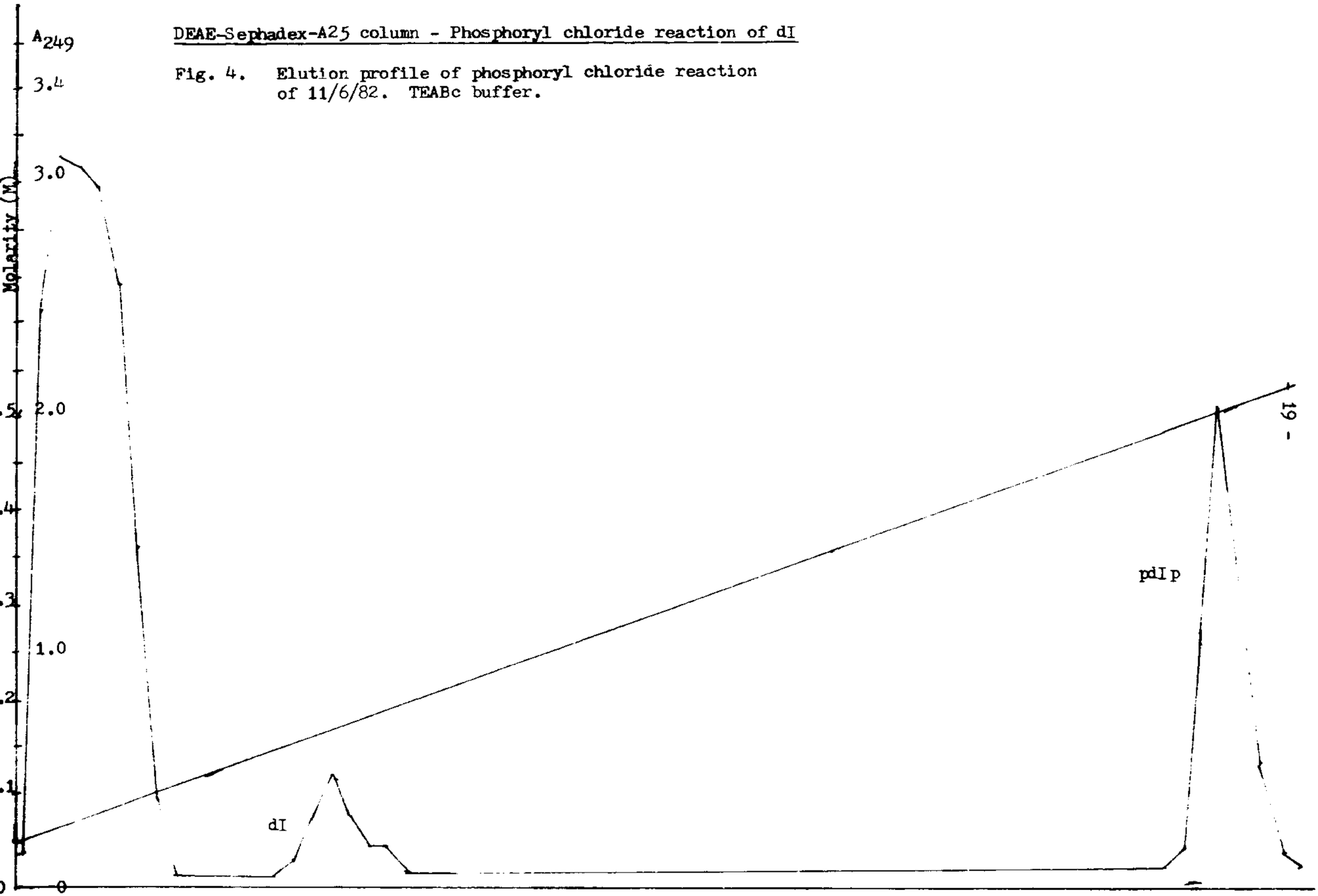


Table 2

Final results of large scale synthesis of pdIp
Phosphoryl chloride reaction of dI

Conditions of synthesis: 0.047 mmoles dI, 1 mmole pyrophosphoryl chloride, -10° - -15°C. Final volume - 250ul. Stopped reaction after 4 hours and 20 minutes. 11/6/82.

	250/260	280/260
Actual ratios	1.54	0.30
Calculated ratios	1.68	0.25

Purity on the HPLC - 2 peaks, 30.8% and 66.2%
Could not determine what the two peaks were. Months later when I checked it again, ther was only one peak and it reacted just like other pdIp I had made.

Total amount of compound synthesized - 10.11mmoles.

DEAE-Sephadex-A25 column - Large scale pdAp deamination reaction

Fig. 5. Elution profile of large scale reaction of 1/18/83. TEAAC buffer.

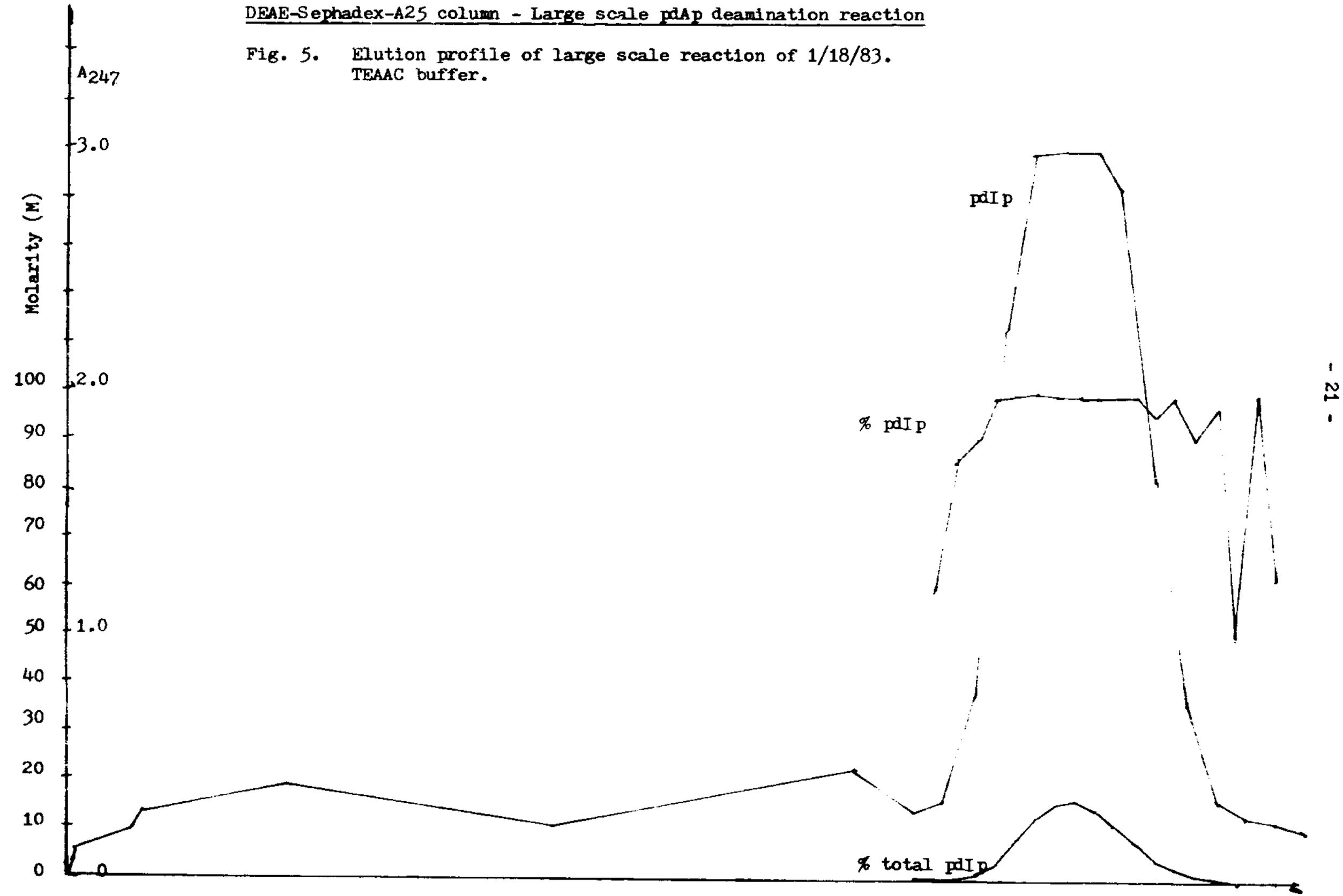


Table 3

Final results of large scale synthesis of pdIp
Deamination of pdAp

Conditions of synthesis: 3.6M NaOAc:HOAc buffer, pH 3.75, .4 mmoles $NaNO_2$, 0.02 mmoles pdAp. Kept $NaNO_2$ concentration at 0.73M. Kept at 5°C. Stopped reaction at 45 hours.

	250/260	280/260	290/260
Actual ratios	1.70	0.28	0.05
Calculated ratios	1.68	0.25	0.03

Absorbance peak at 248nm.

Purity on the HPLC - 98.8%.

Total amount of pdIp synthesized - 9.20 mmoles.

RNA Ligase pilot reactions - rA_3 + pdGp

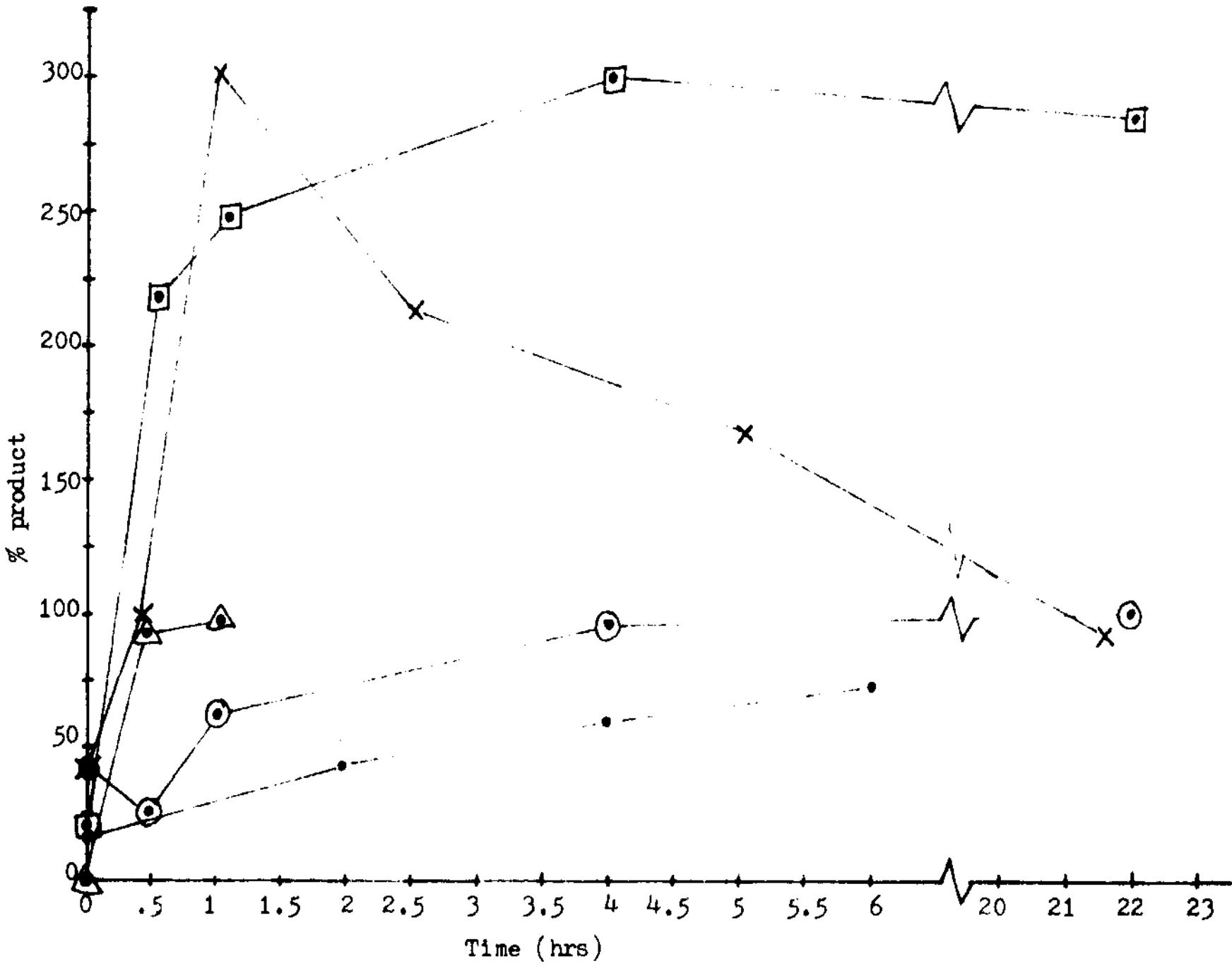


Fig. 6. RNA ligase pilot reactions. Standard conditions: 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM DTT, 2 mM pdGp, 0.5 mM rA_3, 1 mM ATP. All reactions followed by radioactivity.

- ⊙ 20 mM $MgCl_2$, 4 mM spermine, 15.3 uM RNAL, 3/23/82.
- [•] 10 mM $MgCl_2$, 10 mM $MnCl_2$, 15.3 uM RNAL, 3/23/82.
- △ 20 mM $MgCl_2$, 15.3 uM RNAL, 3/23/82
- X 10 mM $MgCl_2$, 10 mM $MnCl_2$, 4 mM spermine, 17.67 uM RNAL, 7/28/82.
- • 10 mM $MgCl_2$, 10 mM $MnCl_2$, 17.67 uM RNAL, 4/6/82.

RNA Ligase pilot reactions - rA_3 + pdGp

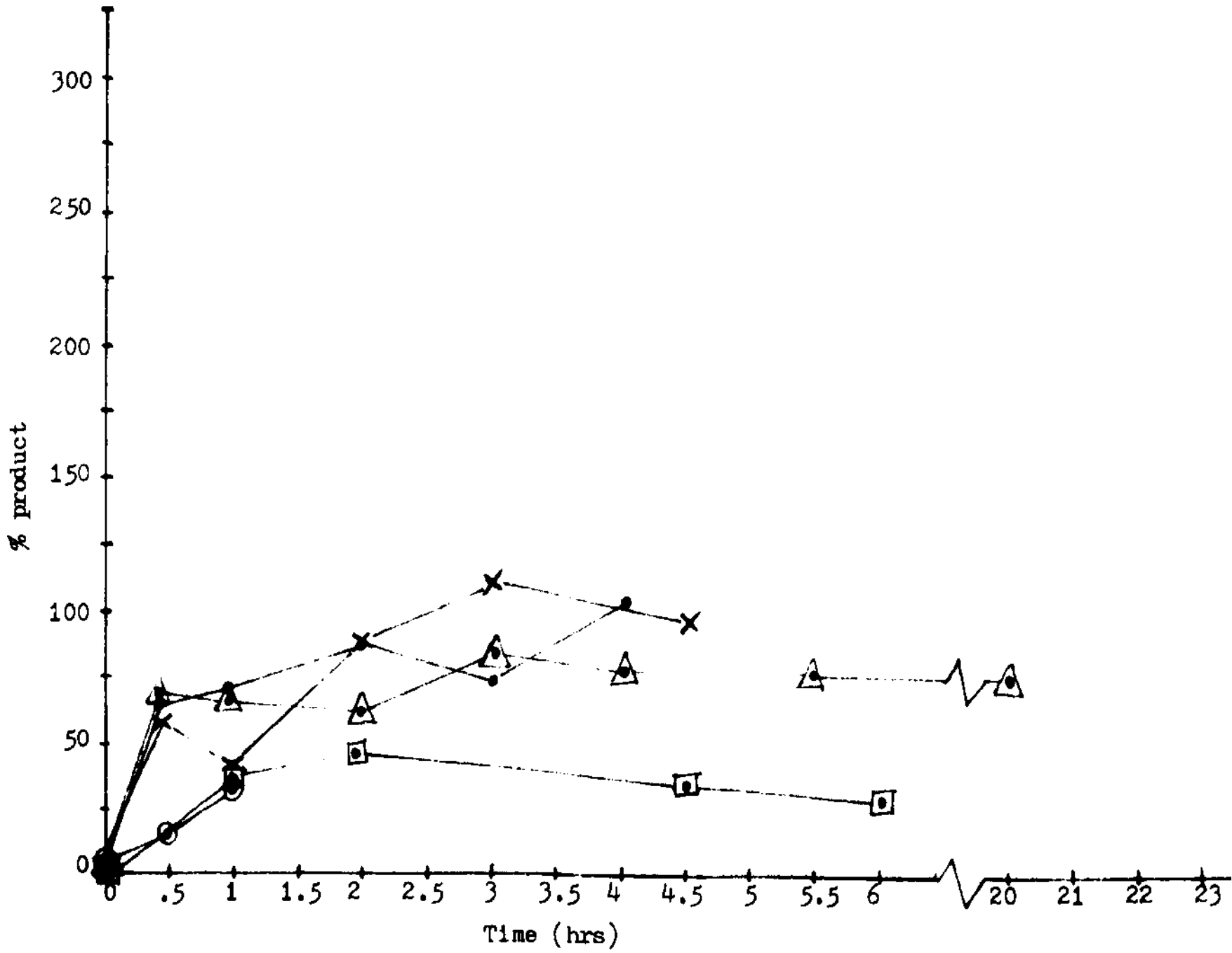


Fig. 7. RNA ligase pilot reactions. Standard conditions: 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM DTT, 2 mM pdGp, 0.5 mM rA_3, 1 mM ATP. ⊙, ⊡ monitored by HPLC and △, ✕, • monitored by radioactivity.

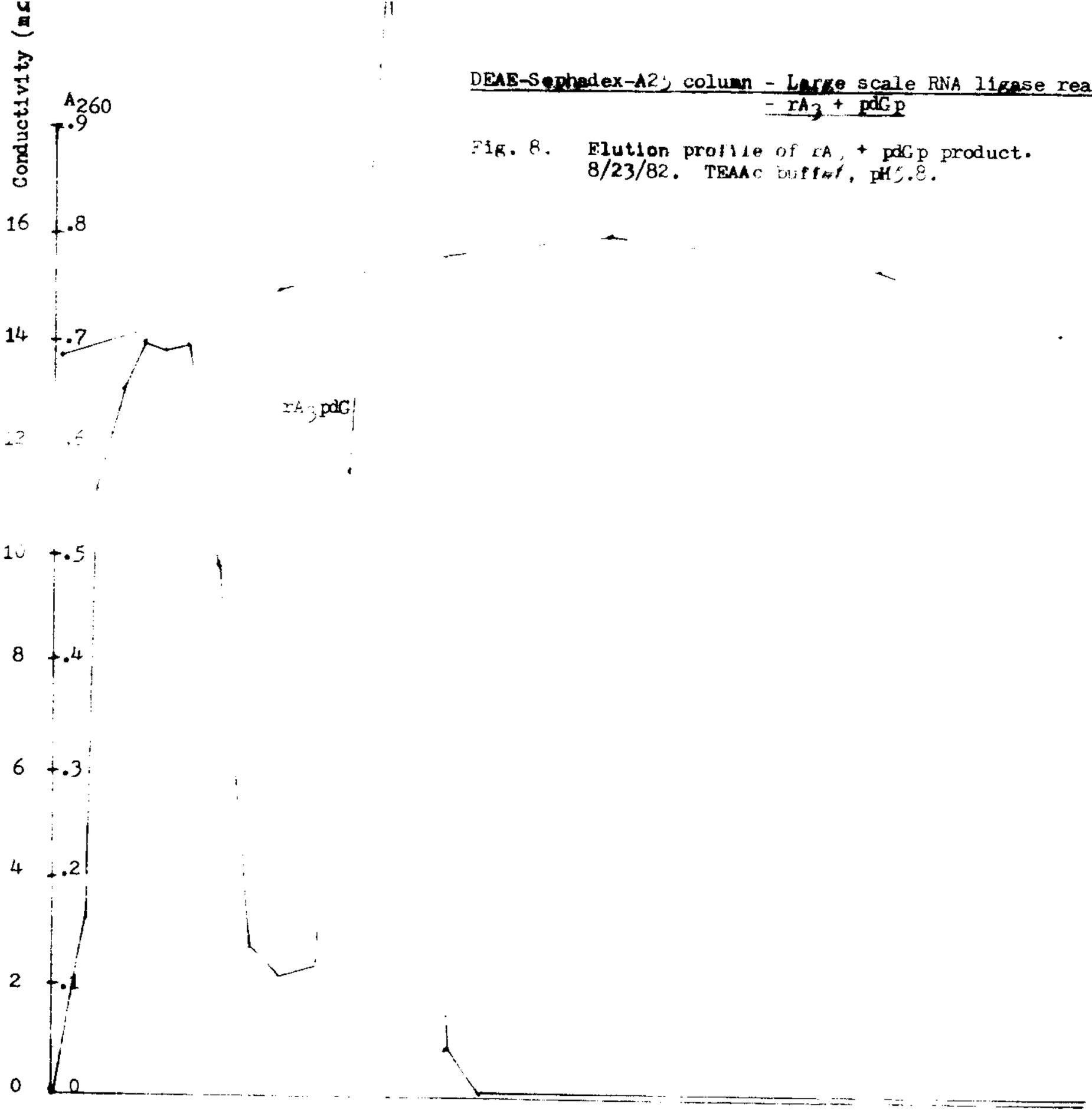
⊙ 10 mM $MnCl_2$, 10 mM $MgCl_2$, 17.67 uM RNAL, 8/3/82.

⊡ 10 mM $MgCl_2$, 10 mM $MnCl_2$, 8.84 uM RNAL, 8/4/82.

△ 20 mM $MgCl_2$, 8.84 uM RNAL, 8/9/82.

✕ 20 mM $MgCl_2$, 8.84 uM RNAL, (checked concentration of stock pdGp solution because I calculated it incorrectly originally) 8/11/82.

• 20 mM Mg Cl_2, 4mM pdGp, 1 mM rA_3, 2 mM ATP, 8.84 uM RNAL, 8/11/82.



DEAE-Sephadex-A25 column - Large scale RNA ligase reaction - rA_3 + pdGp

Fig. 8. Elution profile of rA_3 + pdGp product. 8/23/82. TEAAc buffer, pH 7.8.

Table 4

Final results on large scale synthesis of rA_3pdC

Conditions of synthesis: 4 mM pdCp, 1 mM rA_3, 2 mM ATP, 50 mM Hepe[illegible]fer, pH 8.0, 10 ug/ml BSA, 20 mM $MgCl_2$, 10 mM DTT, 9 uM RNAL (Prep XXVIII), 37°C. Final volume - 200ul. Entire reaction BAPed with 800ul 1 mg/ml BAP. Reaction monitored by the HPLC. Reaction stopped after 4 hours. 8/23/82.

	250/260	270/260	280/260
Actual ratios	.96	.73	.40
Calculated ratios	.89	.69	.29

Absorbance peak at 257nm.

Purity on the HPLC - 89.5%

Total amount of rA_3pdC synthesized - 193 nmoles.

DEAE-Sephadex-A25 column - Large scale RNA ligase reaction

Fig. 9. Elution profile of rA_3 + pdGp product.
TEABc buffer, pH 7.5. 10/14/82.

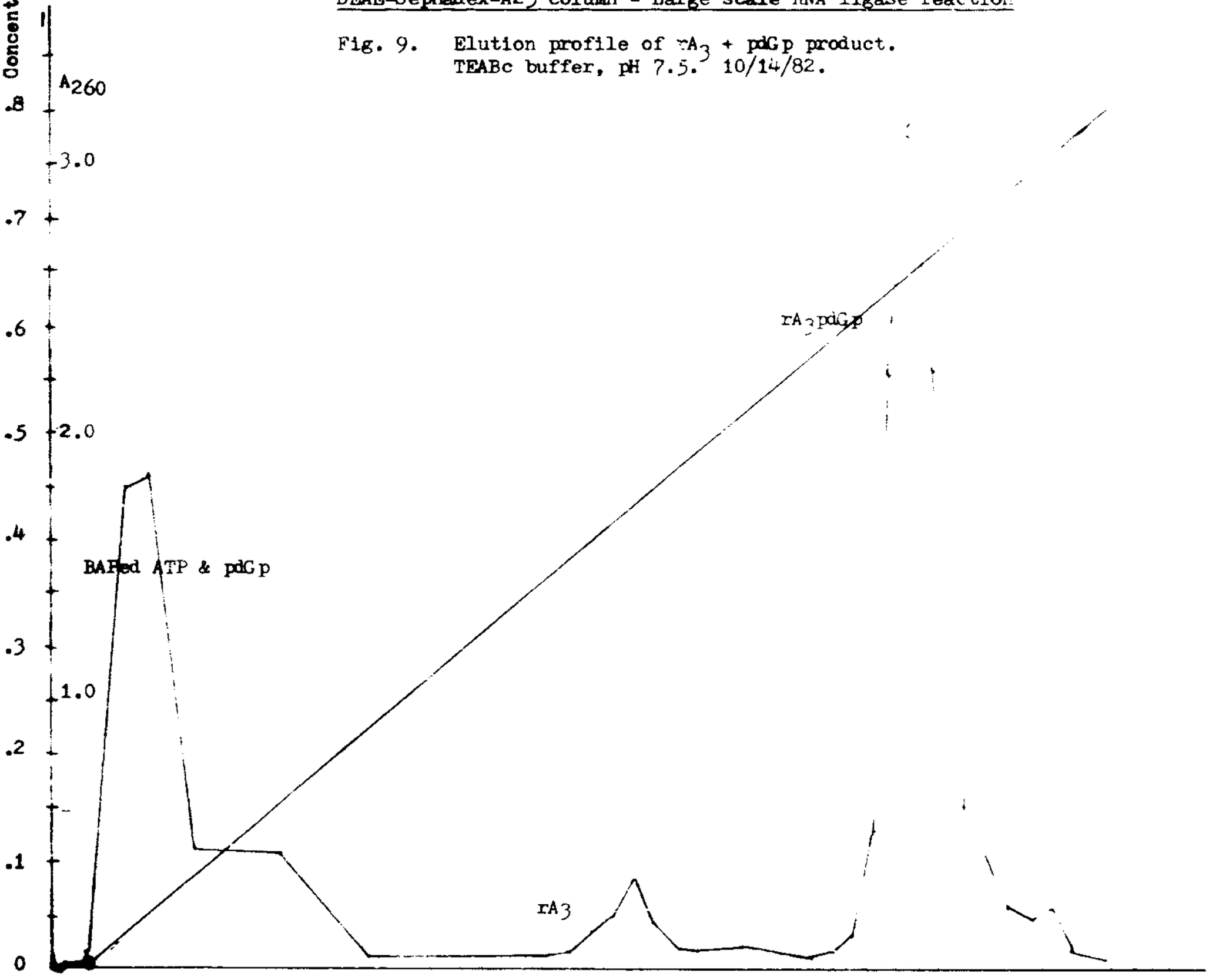


Table 5

Final results of large scale synthesis of rA_3pdGp

Conditions of synthesis: 4 mM pdGp, 1 mM rA_3, 2 mM ATP, 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 20 mM $MgCl_2$, 10 mM DTT, 9 uM RNAL (Prep XXVIII), 37°C. Final volume - 1000ul. Entire reaction BAPed with 2000ul 1 mg/ml BAP. Stopped reaction at 3 hours and 35 minutes. 10/14/82.

	250/260	270/260	280/260
Actual ratios	.93	.75	.40
Calculated ratios	.89	.69	.29

Absorbance peak at 258 nm.

Purity on the HPLC - 98%.

Total amount of rA_3pdG synthesized - 900 nmoles.

RNA Ligase pilot reactions - rA_3pdG + pdIp

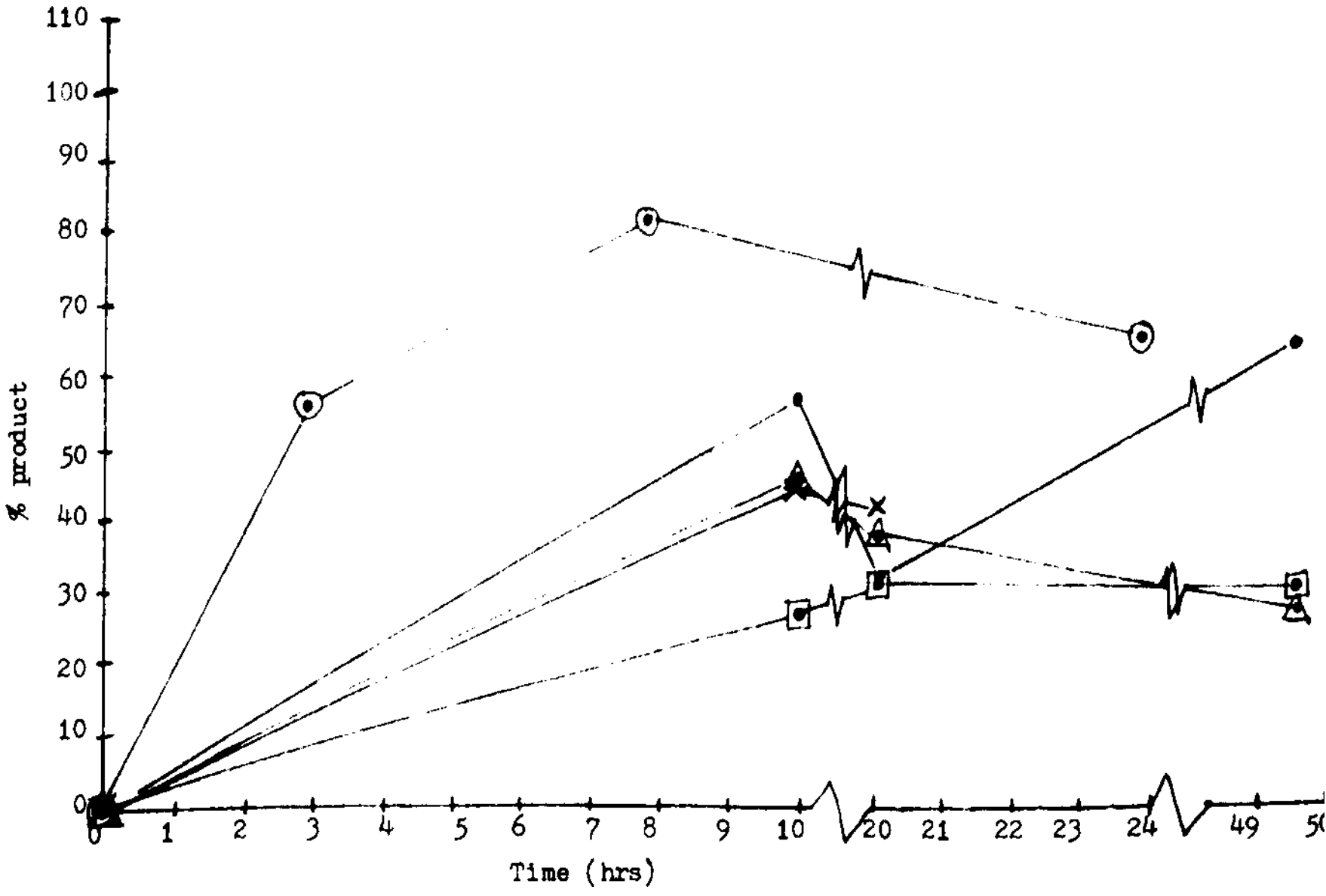


Fig. 10. RNA ligase pilot reactions. Standard conditions: 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM DTT, 2 mM pdIp, 0.5 mM rA_3pdGp, , 37°C. All reactions monitored on the HPLC.

⊙ 16 mM $MgCl_2$, 4 mM $MnCl_2$, 19.68 uM RNAL, 8/7/82, 1 mM ATP.

⊡ 0.5 mM ATP, 10 mM $MgCl_2$, 22.1 uM RNAL, 9/14/82. } Conditions were the same by mistake.

△ 0.5 mM ATP, 10 mM $MgCl_2$, 22.1 uM RNAL, 9/14/82. }

• 0.5 mM ATP, 10 mM $MgCl_2$, 4 mM $MnCl_2$, 13.26 uM RNAL, 9/14/82. } Conditions were the same by mistake.

× 0.5 mM ATP, 10 mM $MgCl_2$, 4 mM $MnCl_2$, 13.26 uM RNAL, 9/14/82. }

RN: Ligase pilot reactions - rA_3pdG + pdIp

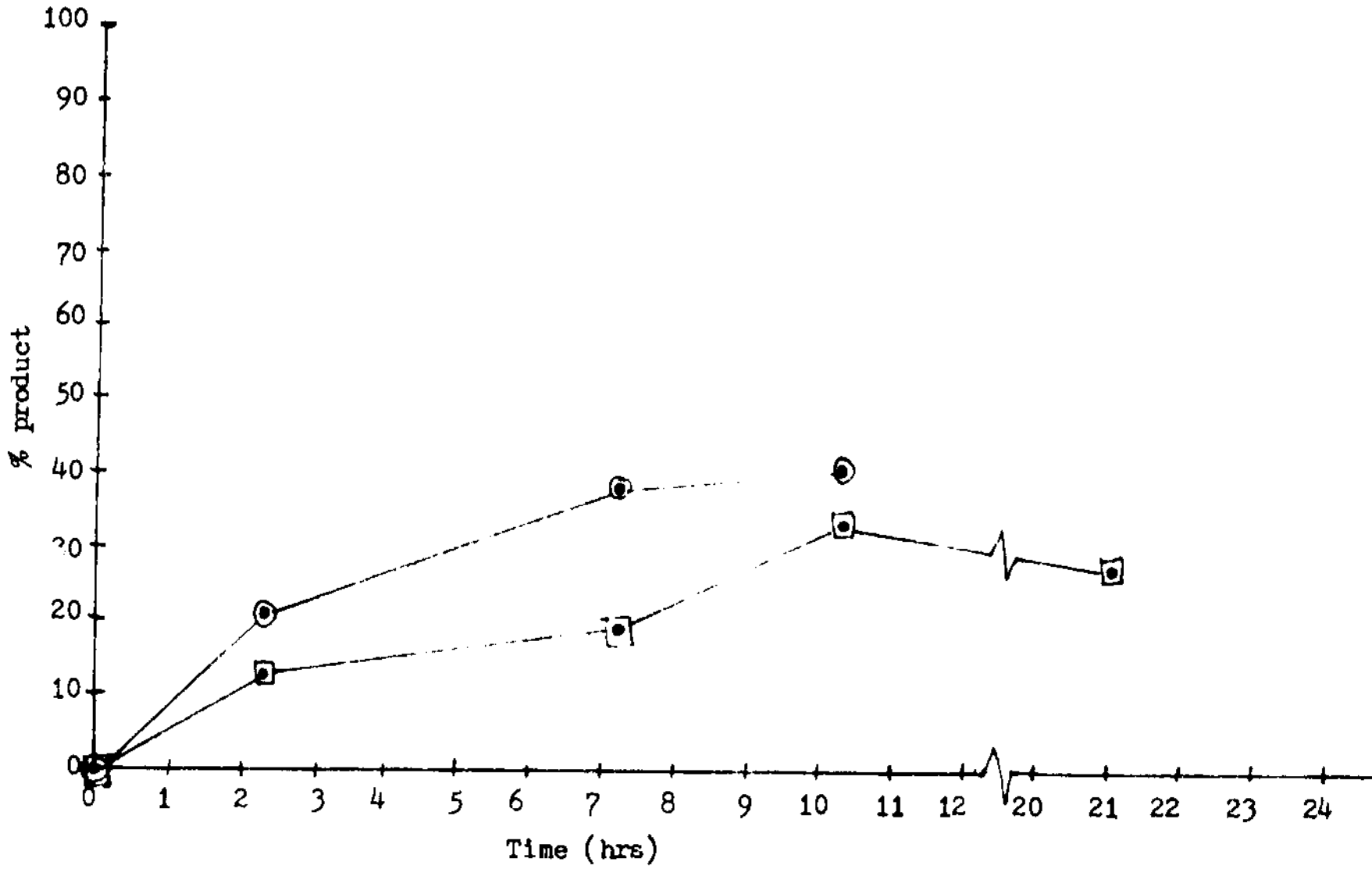


Fig. 11. RNA ligase pilot reactions. Standard conditions: 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM DTT, 4 mM pdIp, 0.5 mM rA_3pdG, 1 mM ATP, 37°C, All reactions monitored by the HPLC.

◉ 10 mM $MgCl_2$, 4 mM $MnCl_2$, 22.1 uM RNAL, 9/28/82.

⊡ 10 mM $MgCl_2$, 4 mM $MnCl_2$, 13.26 uM RNAL, 9/28/82.

RNA Ligase pilot reactions - rA_3pdG + pdIp

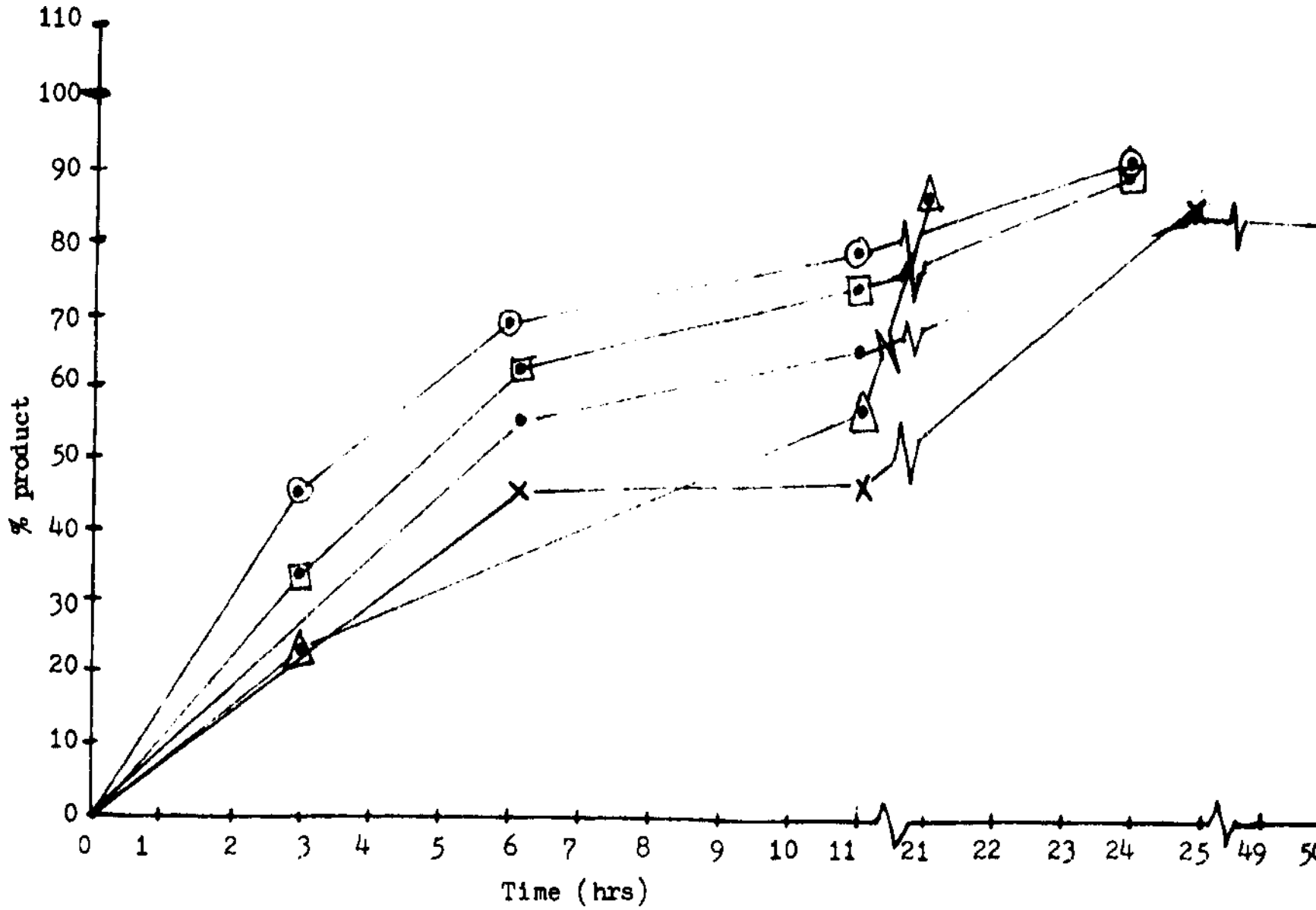
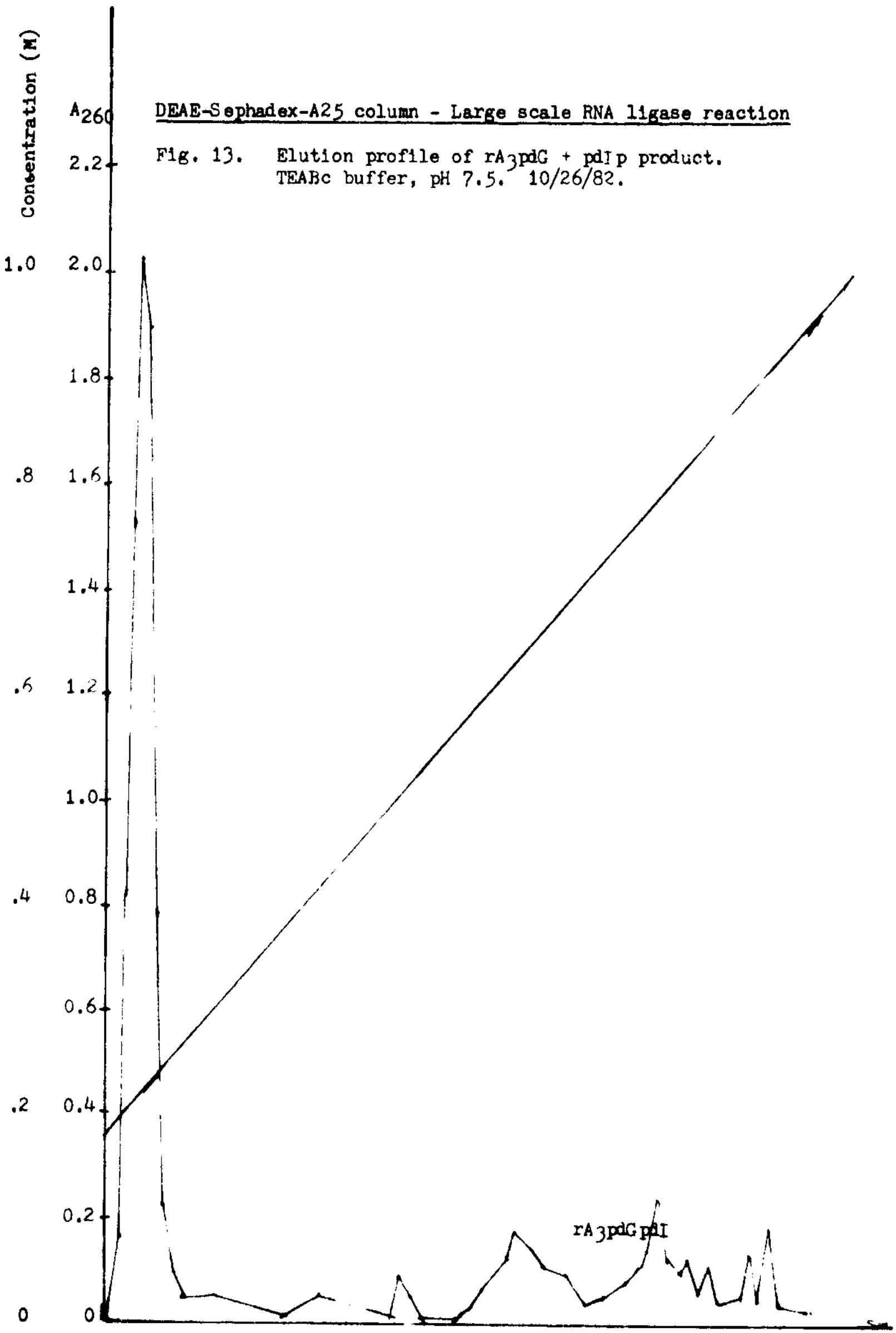


Fig. 12. RNA ligase pilot reactions. Standard conditions: 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM DTT, 4 mM pdIp, 0.5 mM rA_3pdG, 1 mM ATP. All reactions monitored by the HPLC.

- ◉ 10 mM $MgCl_2$, 5 mM $MnCl_2$, 15% DMSO, 17°C, 22.79 uM RNAL, 10/6/82.
- ⊡ 10 mM $MgCl_2$, 5 mM $MnCl_2$, 15% DMSO, 17°C, 11.40 uM RNAL, 10/6/82.
- △ 5 mM $MnCl_2$, 17°C, 22.79 uM RNAL, 10/6/82.
- • 10 mM $MgCl_2$, 5 mM $MnCl_2$, 17°C, 22.79 uM RNAL, 10/6/82.
- X 10 mM $MgCl_2$, 5 mM $MnCl_2$, 22.79 uM RNAL, 10/6/82, 4°C.



DEAE-Sephadex-A25 column - Large scale RNA ligase reaction

Fig. 13. Elution profile of rA_3pdG + pdIp product. TEABc buffer, pH 7.5. 10/26/82.

DEAE-Sephadex-A25 column - Second purification of large scale RNA ligase reaction

Fig. 14. Elution profile of peak from large scale rA_3pdG + pdIp reaction of 10/26/82. TEABc buffer, pH 7.5.

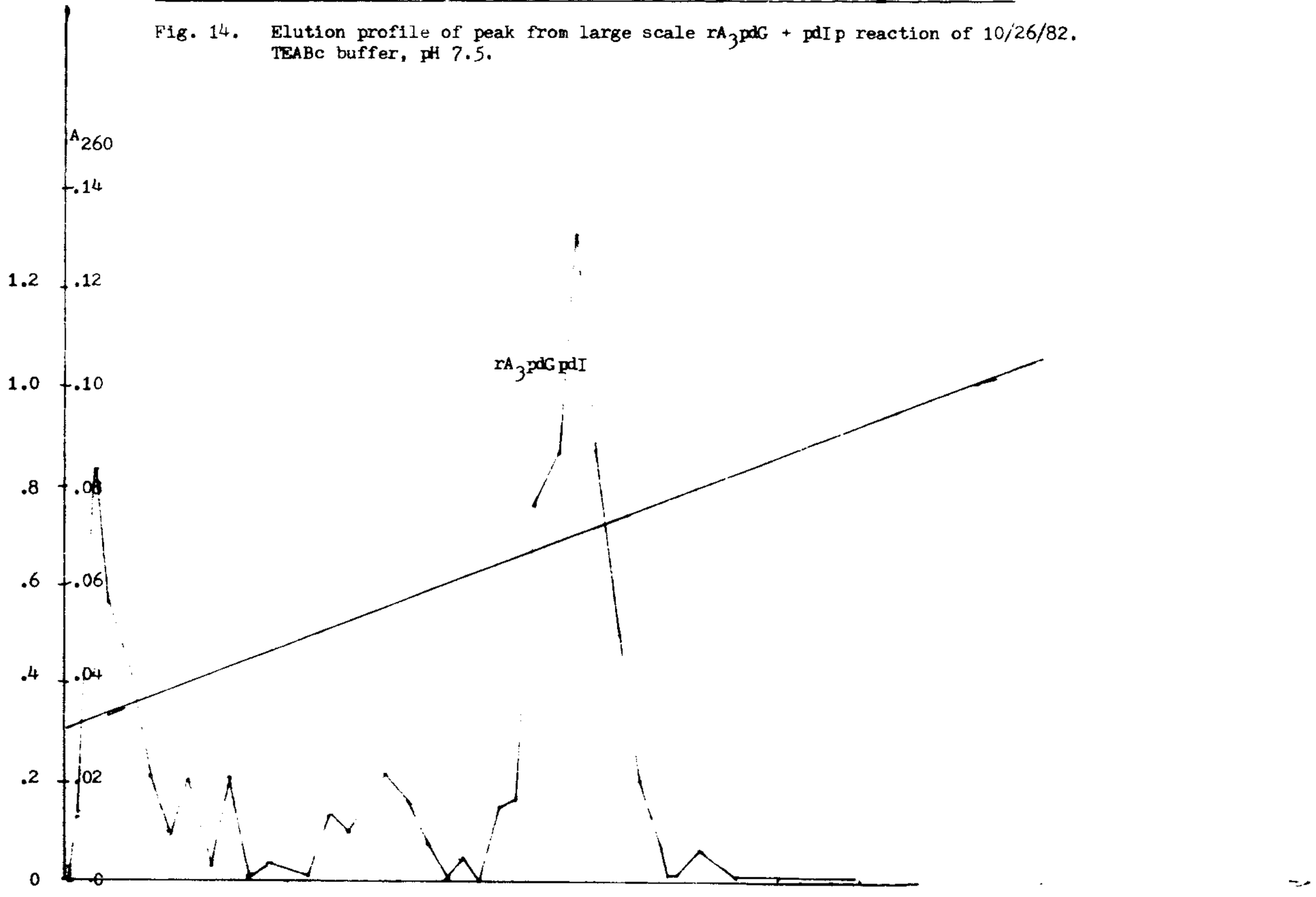


Table 6

Final results of large scale synthesis of $rA_3pdGpdI$

Conditions of synthesis: 4 mM pdIp, 0.5 mM rA_3pdG, 1 mM ATP, 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM DTT, 15% DMSO, 11.40 uM RNAL (Prep XXVIII), 17°C. Final volume - 350 ul. Stopped reaction after 42 hours and 5 minutes. BAPed entire reaction with 700 ul 1mg/ml BAP. 10/26/82.

	250/260	270/260	280/260
Product ratios	.99	.67	.33
Calculate ratios	1.05	.61	.22

Absorbance peak at 255.5 nm.

Nucleoside analysis :

Standard:	rA:dG:dI	3.19:1:1.09
Product:	rA:dG:dI	2.60:1:.57

Purity on the HPLC (Zorbax) - 96.1%.

Total amount of $rA_3pdGpdI$ synthesized - 16.5 nmoles.

RNA Ligase pilot reactions - rA_3pdG + pdIp

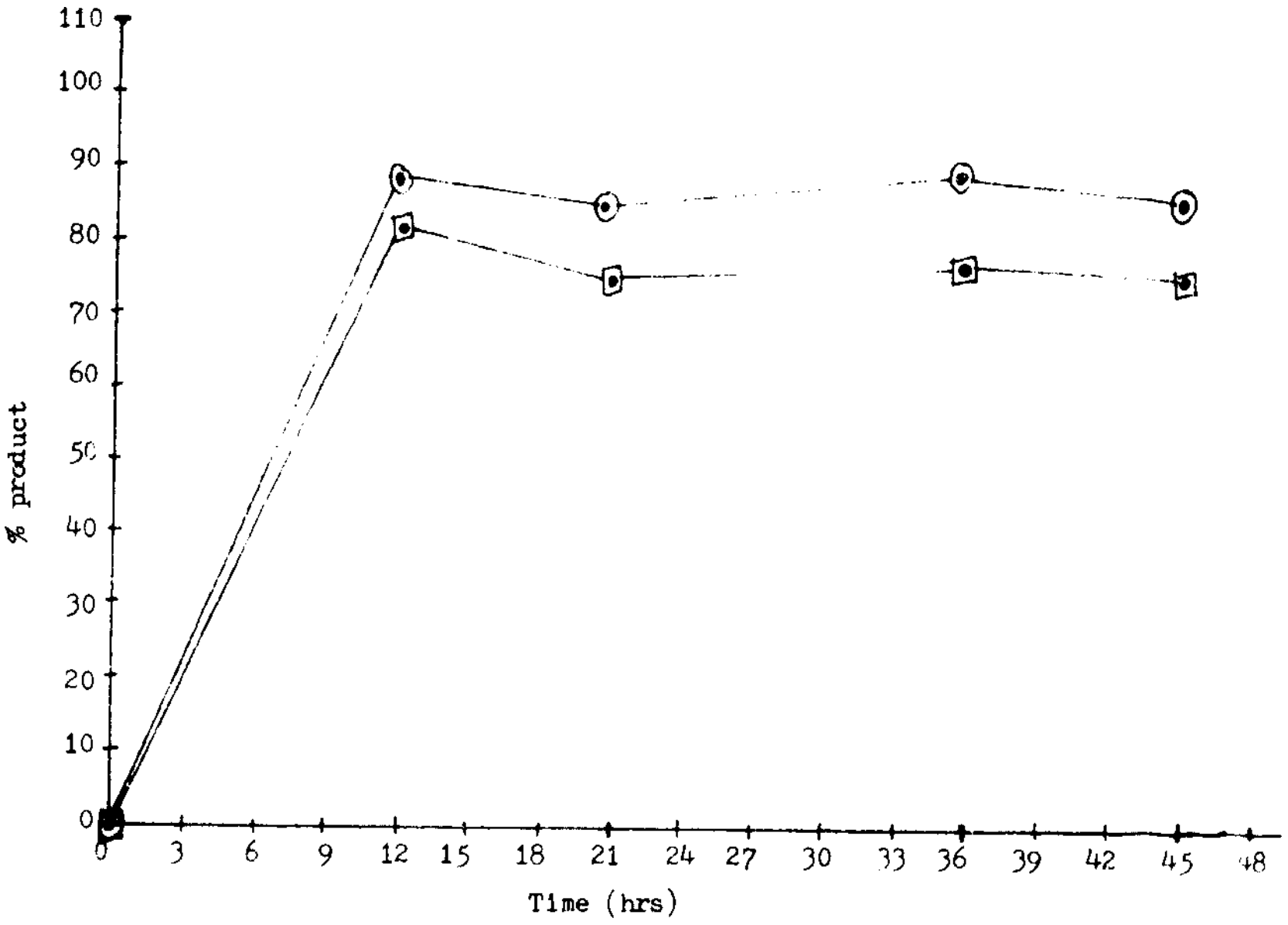


Fig. 15. RNA ligase pilot reactions. Standard conditions: 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM DTT, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 15% DMSO, 17°C, 11.40 uM RNAL, 1/28/83. All reactions monitored by the HPLC.

⊙ 4 mM pdIp, 0.5 mM rA_3pdG, 1 mM ATP.

⊡ 8 mM pdIp, 1 mM rA_3pdG, 2 mM ATP.

RNA Ligase pilot reactions - rA_3pdG + pdIp

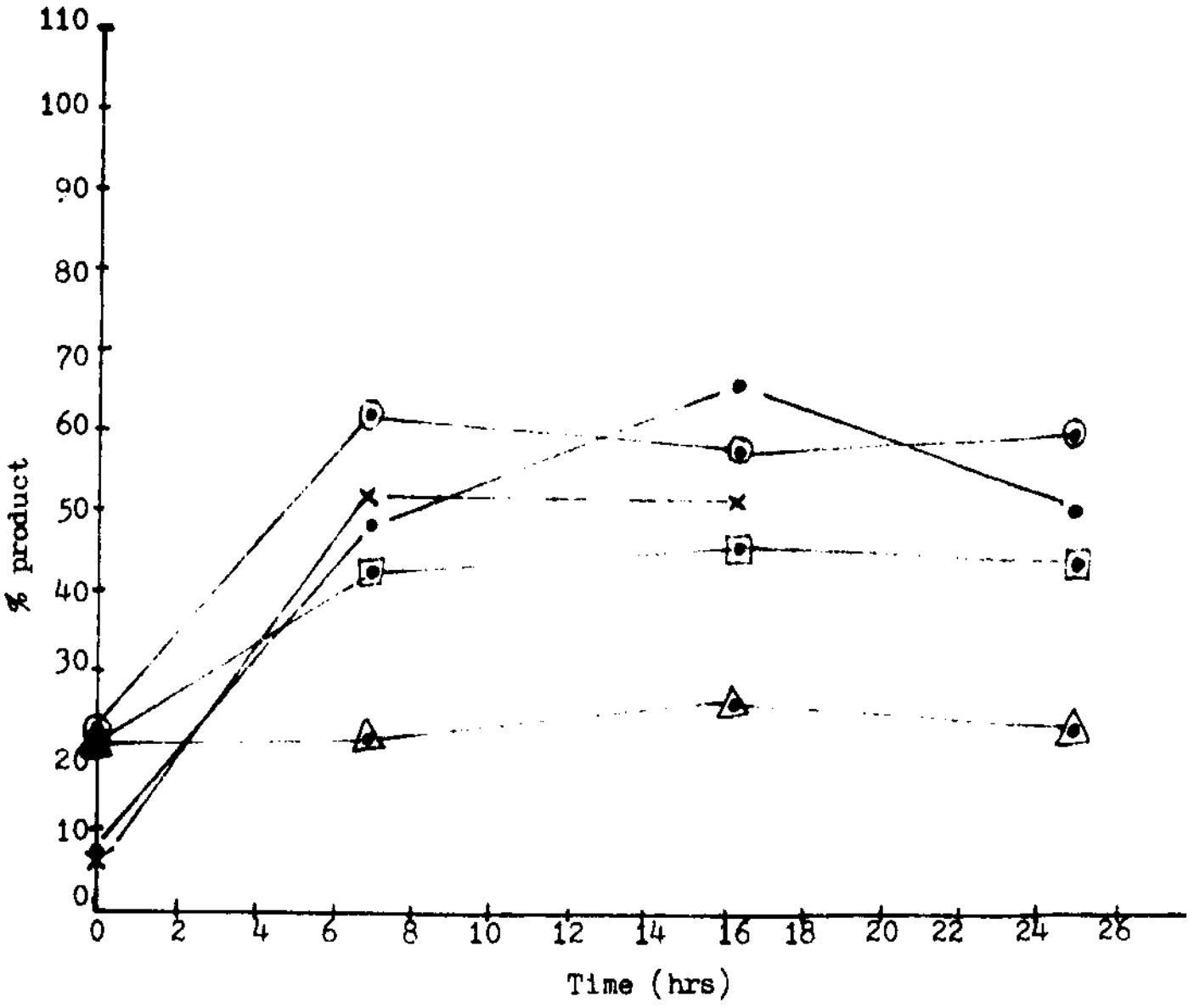
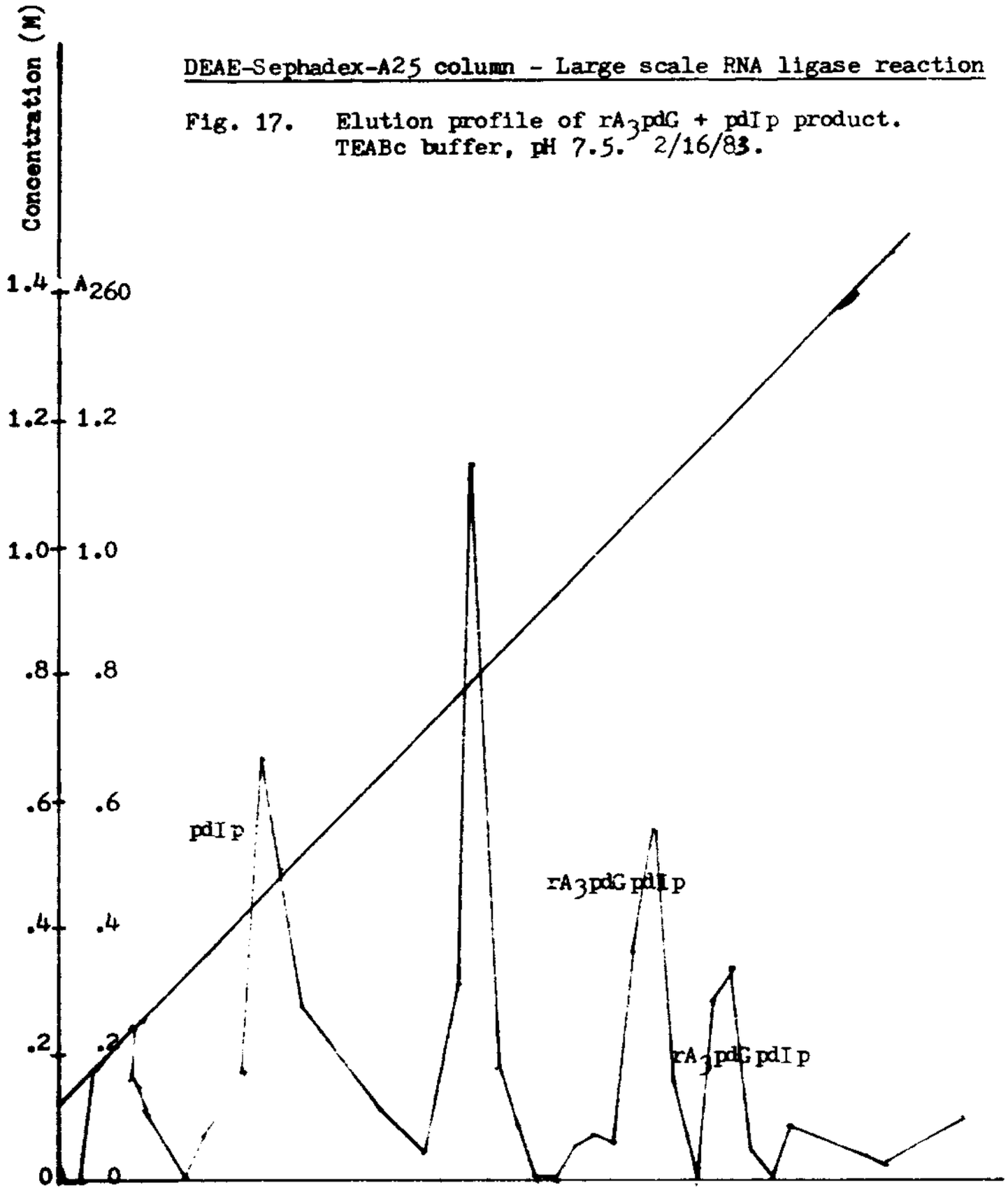


Fig. 16. RNA ligase pilot reactions. Standard conditions: 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM DTT, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 17°C, 11.40 uM RNAL, 2/2/83. All reactions monitored by the HPLC.

- ◎ 8 mM pdIp, 1 mM rA_3pdG, 2 mM ATP.
- ⊡ 8 mM pdIp, 1 mM rA_3pdG, 1 mM ATP.
- △ 8 mM pdIp, 1 mM rA_3pdG, 0.5 mM ATP.
- • 8 mM pdIp, 1 mM rA_3pdG, 0.4 mM ATP, 1 mM phosphocreatine, 1 ul myokinase, 1 ul phosphocreatine kinase (ATP regeneration system)
- X 8 mM pdIp, 1 mM rA_3pdG, 1 mM ATP (used pdIp made 11/6/82 - thought it was two peaks and then reverted)

DEAE-Sephadex-A25 column - Large scale RNA ligase reaction

Fig. 17. Elution profile of rA_3pdG + pdIp product.
TEABc buffer, pH 7.5. 2/16/83.



DEAE-Sephadex-A25 column - Second purification of large scale RNA ligase reaction

Fig. 18. Elution profile of two combined $rA_3pdGpdI$ peaks from RNA ligase reaction of 2/16/83. TEABc buffer, pH 7.5.

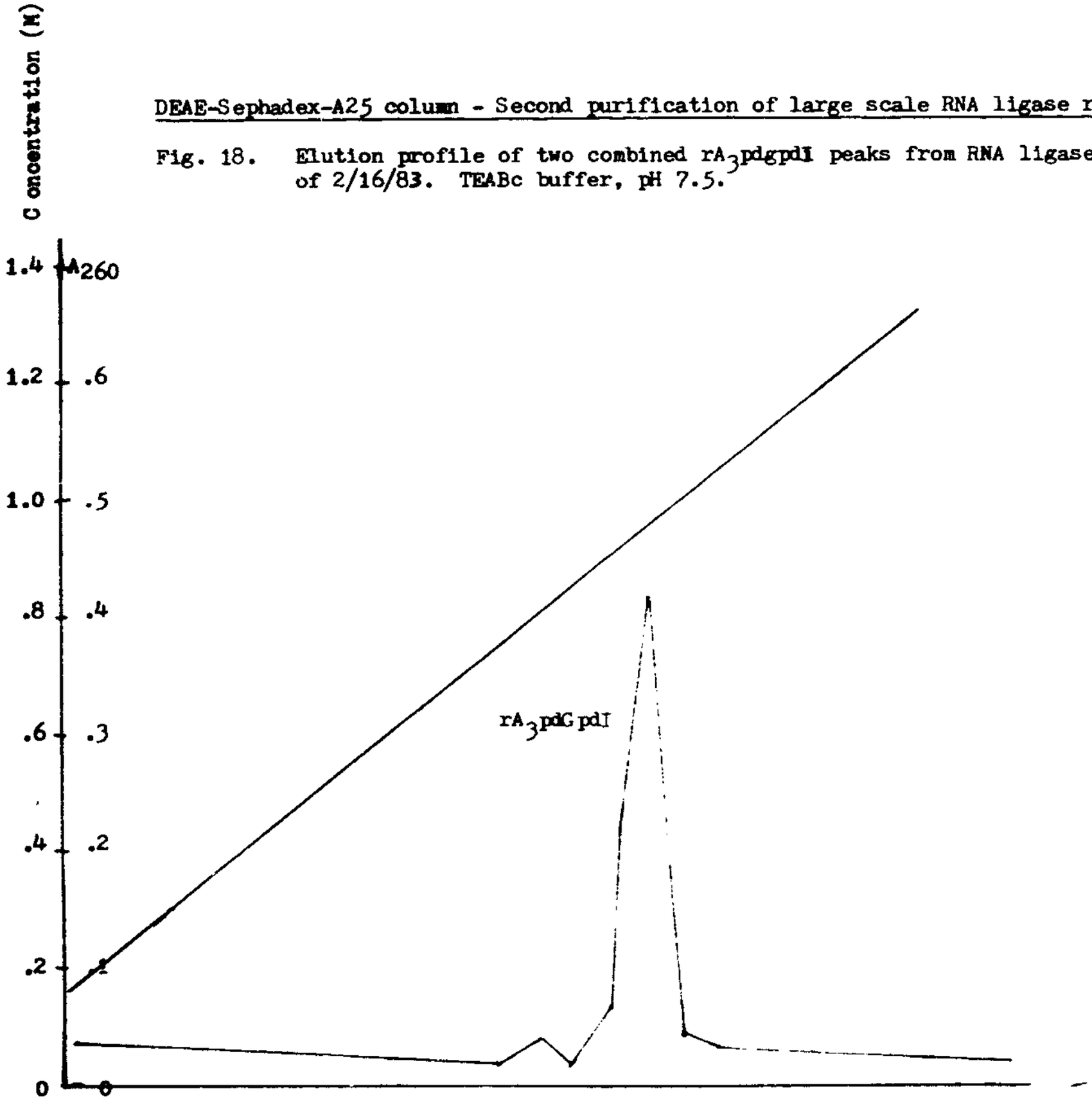


Table 7

Final results of large scale synthesis of $rA_3pdGpdI$

Conditions of synthesis: 8 mM pdIp, 1 mM rA_3pdG, 2 mM ATP, 50 mM Hepes buffer, pH 8.0, 10 ug/ml BSA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM DTT, 15% DMSO, 17°C, 11.40 uM RNAL. Stopped reaction after six hours. Did not BAP entire reaction. 2/16/83.

	250/260	270/260	280/260
Product ratios	1.00	.68	.33
Calculated ratios	1.05	.61	.22

Nucleoside analysis:

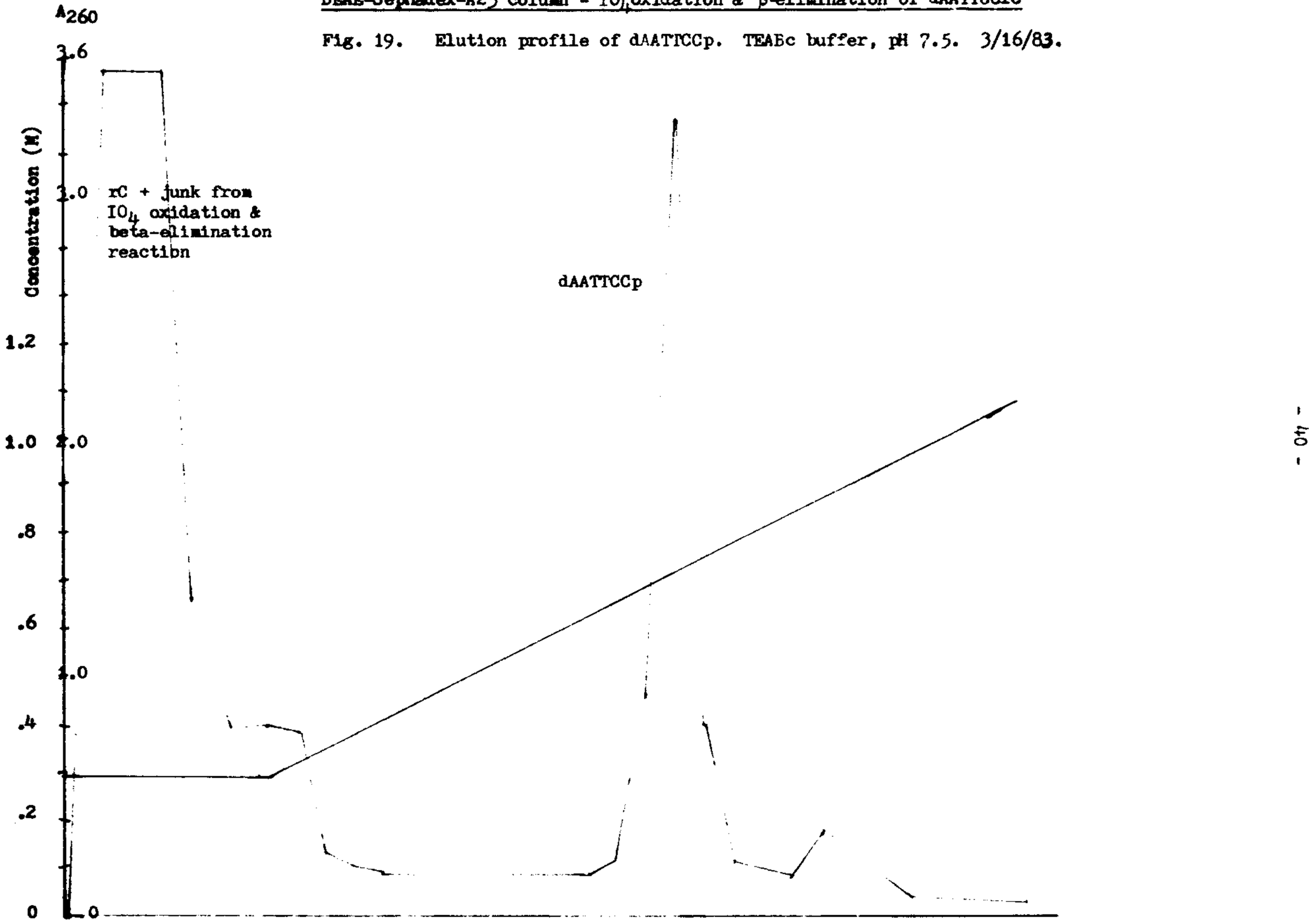
Standards	rA:dG:dI	2.72:1:.92
$rA_3pdGpdI_1$	rA:dG:dI	3.16:1:.62
$rA_3pdGpdI_2$	rA:dG:dI	2.65:1:.56

Purity on the HPLC (Zorbax) - 96.4%

Total amount of $rA_3pdGpdI$ synthesized - If both peaks are $rA_3pdGpdI$, I will have synthesized 62 nmoles of product.

DEAE-Sephadex-A25 column - IO_4 oxidation & β-elimination of dAATTCCrC

Fig. 19. Elution profile of dAATTCCp. TEABc buffer, pH 7.5. 3/16/83.



DEAE-Sephadex-A25 column - Final purification of pdAATTCCp

Fig. 20. Elution profile of pdAATTCCp. TEABc buffer, pH 7.5 - 8.0. 4/15/83.

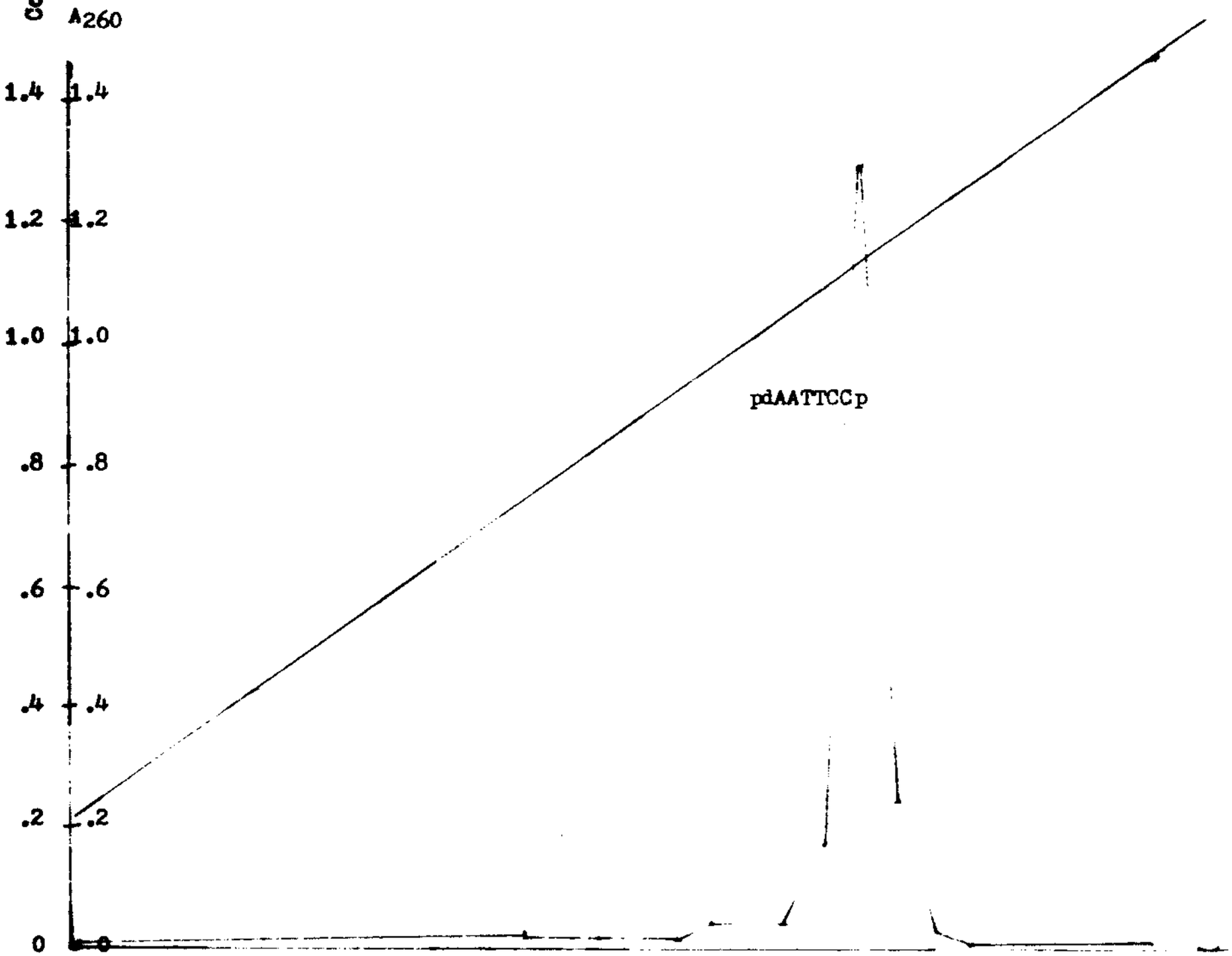


Table 8

Final results of reactions involving dAATTCCrC

Reactions performed involving dAATTCCrC: Periodate oxidation and beta-elimination and phosphate kinase reaction.

	250/260	270/260	280/260
Product ratios	.78	.92	.60
Calculated ratios	.76	.99	.61

Nucleoside analysis:

Standards	rC:dC:dT:dA	1:1.84:1.80:1.98
Product	rC:dC:dT:dA	1:2.31:2.53:2.52

Purity on the HPLC (Zorbax) - 96.8%

Amount of pdAATTCCp synthesized - 153 nmoles

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