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ENTITLED Nucleotide Phosphotransferase: Determination of

Linear Range and Survey of Donors

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NUCLEOTIDE PHOSPHOTRANSFERASE:
DETERMINATION OF LINEAR RANGE AND SURVEY OF DONORS

BY

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List of Abbreviations

T		2' - deoxythymidine
pT		2' - deoxythymidine - 5 - phosphate
pTp		2' - deoxythymidine - 3' - 5' - bisphosphate
*T	[2-^{14}C]	2' - deoxythymidine
p*T	[2-^{14}C]	2' - deoxythymidine - 5' - phosphate
p*Tp	[2-^{14}C]	2' - deoxythymidine - 3' - 5' - bisphosphate
pNØp, $pNØPO_4$		p - nitrophenylphosphate
pNØ		p - nitrophenol
3'-dGMP		2' - deoxyguanosine - 3' - phosphate
2'-AMP		adenosine - 2' - phosphate
PEP		Phosphoenolpyruvate

Introduction

In 1970, Elinor F. Brunngraber and Erwin Chargaff isolated an enzyme from E. coli that catalyzed the transfer of phosphate from various organic donors to the free 2' and 2' hydroxyls of nucleoside and nucleotide acceptors (1). Because nucleotides were found to accept transferred phosphate more readily than nucleosides, the enzyme was called nucleotide phosphotransferase (2). Among the many compounds surveyed by Brunngraber and Chargaff pNØp and 3'-dGMP were reported as among the most efficient donors (3). The enzyme is also able to transfer the phosphate to the 3'-hydroxyl of oligonucleotides. Thymidine oligomers up to six residues long have been found that accept phosphate from donors at their 3'-terminal hydroxyl in a reaction catalyzed by nucleotide phosphotransferase (4).

The biological function of this enzyme is not known. Brunngraber and Chargaff have shown that nucleotide phosphotransferase will phosphorylate NAD^+ to form NADP. (5) This has lead to speculation that the enzyme could be an alternative to NAD kinase and ATP in synthesizing NADP. Another possible function is seen in the ability of nucleotide phosphotransfrase to phosphorylate the 3'-hydroxyl of thymidine oligomers. With many cellular processes requiring a free 3'-hydroxyl, nucleotide phosphotransfrase could be acting as regulatory agent. Some

of processes that would be inhibited by a lack of 3'-hydroxyl include the changing of t-RNA, the replication of DNA and the transcription of RNA.

The ability of nucleotide phosphotransfrase to phosphorylate the 3'-hydroxyl could make it a useful tool in experiments that require the in-vitro phosphorylaton of oligonucleotides at the 3'-hydroxyl. Possible uses of this would include labeling for sequencing or binding studies and the protection of 3'-hydroxyl by phosphosphorylation during T4 RNA ligase catalyzed synthetic reactions (6). The ability to use nucleotide phosphotransfrase to accomplish 3'-phosphorylaton would be a significant improvement because current methods of 3'-phosphorylation are complicated. They involve the addition of a single 5'-labelled ribonucleotide to the 3'-hydroxyl of the oligmer using various enzymatic methods or by chemical synthesis and then removing the ribonucleotide by periodate oxidation followed by beta-elimination to leave the desired 3'-labelled product (7). If 3'dGMP should be an excellent donor for this enzyme, it could provide an easily obtainable source of labelled 3'-phosphates due to the availability of labelled [3'-^{32}P]dGMP. Labelled 3'-dGMP can be synthesized from [α-^{32}P] dGTP, which is obtainable commercially. First [α-^{32}P]GTP would be polymerized into a poly(dG) by deoxynucleotide terminal transferase which will add [α-^{32}P]dGTP onto a

oligodeoxyguanosine primer. The poly (dG) chain would then be cleaved by DNase II and spleen phosphodiesterase to yield the desired product, [3'-^{32}P] dGMP (8).

The focus of this work is to identify a linear range for the assay of the enzyme and using that information to survey a wide range of potential donors to find one with a low K_m. If a donor is found with a sufficiently low K_m, nucleotide phosphotransfrase could become a useful tool in laboratory biochemistry.

Materials and Methods

Materials. *E. coli* W397e cells were supplied by Charles Pratt of the University of Illinois Microbiology Department. Nucleotide phosphotransferase was isolated from *E. coli* by Steven Marquez using a purification scheme developed in this lab (9). pNOp was supplied by Aldrich Chemicals. The 3'-dGMP was from Pharmacia. All other donors surveyed were from Sigma Corporation. The [2'-^{14}C] 2'-deoxythymidine-5'-phosphate was from ICN. Thin layer chromatograms, type PEI-300, were obtained from Brinkmann Instruments. The TLC sheets were scanned in the radio thin-layer chromatograph, manufactured by Radiomatic Instruments and Chemical Co., model RS.

Assay

The assay procedure used was based on the following reaction.

$$pN\emptyset p + p^{*}T \text{ ----> } pN\emptyset + p^{*}Tp$$

For the experiments to determine the linear range, the assay contained 100 mM pNOp as the donor, 20 mM $p^{*}T$ as the acceptor and 0.1 M NaOAc (pH 5.0) as the buffer. Different enzyme concentrations were tested in a total reaction volume of 20 ul. Enzyme was diluted in 0.1M NaOAc (pH 5.0) and 10 ul was added to start the reaction.

Each reaction was incubated at 37° C for the desired time. The reaction was stopped by adding 5 ul of 1N KOH. Aliquots (5 ul) of the reaction mixture were spotted onto a 20x20 cm PEI-300 TLC sheet. Each TLC contained a standard lane of 140 nmoles each of T, pT and pTp. The TLC plate was developed by ascending chromatography at 37° C with 0.8M LiCl. The plates were dried with cool air. The nucleotides on the plates were visualized under ultraviolet light and their position marked (Figure 1). The standard lane contained three spots corresponding to pTp, pT and T. An assay lane usually had spots with Rf values corresponding to the pTp and pT standards. The radioactivity in each lane was then quantified by the scanner.

For surveying donors, the assays contained a

concentrated donor solution, 13.33 mM p*T and 0.1 M NaOAc (pH 5.0). The donors were tested at 1500 uM, 500 uM, and 100 uM. The final volume in each assay tube was 30 ul. Some assays had different concentrations of donor, acceptors and other reagents and these conditions are noted where appropriate. Each reaction was incubated at 37°C for the desired time. At the indicated time, an aliquot of 7.5 ul was removed from the tube and added to 2.5 ul of 1N KOH to stop the reaction. An aliquot (5 ul) of this mixture was spotted on the PEI-300 TLC plates. Each plate contained the same standards mentioned previously but the concentration of pTp, pT and T were reduced to 70 nmoles. The lowered concentration improved the resolutions of the standards when they are developed. Also, the TLC plates were developed at 4°C to improve resolution of the standards.

The extent of reaction in each assay, referred to as % pTp, for both studies, was determined from the output of radioactivity as determined by the scanner. Each plate was scanned and by comparing the patterns of ultraviolet absorbances of the TLC plate and the radioactive profile, the identity of each radioactive peak could be determined. The percent pTp was determined by dividing the radioactivity in the pTp peak by that in the pTp and pT peaks. The background in other areas of the chromatogram was

insignificant and was therefore not subtracted from the data.

Results

It was necessary to determine the range of the enzyme concentration over which the assay was linear in order to perform the donor survey. The linear range for nucleotide phosphotransferase was studied previously by Satya Khuon in our laboratory. She reported the assay to be linear between 30 ug/ml and 60 mg/ml (10). The enzyme was tested at concentrations ranging from 2.5 ug/ml and 60 ug/ml. Time points were taken at 0, 3, 7, 13, 20, 30 and 60 minutes. Each reaction was analyzed as described in Methods. The results of these experiments are shown in Table 1 and the graph of those results is shown in Figure 2. The fact that the % pTp produced in Figure 2 does not go through zero can probably be explained by two different factors. First, there is some product formed even when the reactions are run with a boiled enzyme. This shows that the collisions of two p^*T molecules can form p^*Tp. The second factor is the approximately 20-30 seconds that elapses between the time I add the enzyme to start the reaction and the aliquot is added to the KOH to stop the reaction. These two factors probably account for the non-zero value. Based on those results shown, I determined that the linear range for

nucleotide phosphotransferase was from 10 ug/ml to 40 ug/ml. A graph of velocity versus enzyme concentration also indicates this result (Figure 3). Based on these results, I selected 30 ug/ml as the enzyme concentration for use in the survey of donors.

The results of the donor survey will be presented in two parts. The first will be the data obtained from surveying the various donors using a nucleotide phosphotransferase concentration of 30 ug/ml. The second will be the data obtained when, in an effort to produce more $p^{*}Tp$, the concentration of nucleotide phosphotransferase was first raised to 60 ug/ml and then lowered to 45 ug/ml.

As described in the materials section, the procedures for these experiments involved assaying various donors at three different concentrations: 1500 uM, 500 uM and 100 uM and taking measurements at 0, 15 and 60 minutes. Brunngraber and Chargaff had previously performed a donor survey and their results were used to narrow the list of donors to be assayed (11). Because the reaction now had a donor concentration of 1500 uM or less, I performed several assays with 3'-dGMP and pNØp to confirm that the reaction would work at the lower donor concentration and that there would be enough pTp present to successfully, formed from a donor, to detect on the scanner. In the course of doing these trials, I determined that developing the TLC plates at

4°C improved the separation of the nucleotides. Also, reducing the concentration of the standards to 35 mM also improved their resolution. The results of each assay on a donor were compared to a 3'-dGMP standard donor. 3'-dGMP was chosen as the standard because Brunngraber and Chargaff had previously reported it as the best donor. A scan of the results of an assay with 3'-dGMP and another donor are shown in Figures 4 and 5. As can be seen, the amount of $p^{*}Tp$ is greater in the assay using 3'-dGMP as the donor, the amount of product formed is not very great. The results for this donor survey are presented in Table 3. The results with each donor are presented normalized with respect to 3'-dGMP at each surveyed concentration and at each time point. The results showed that the best donors, listed from best to worst are: 3'-dGMP, 2'-AMP, Acetyl Coenzyme A(Li^{+} salt), PEP, Phosphatidyl choline, Phosphocreatine, pNØp, thymidine-3', 5'-diphosphate and Fructose-1, 6-diphosphate. Inactive donors were 0-phosphoserine, Ribose-1-phosphate and Tyrosine-phosphate. Since only a small amount of product was produced it was difficult to make meaningful comparisons between donors. As a result, it was suggested to increase the extent of the reaction so that the % pTp produced would be approximately 20% after 15 minutes of reaction. As initially performed (Table 3), the maximum yield of pTp was 12 percent after 60 minutes. Also, the amount of

radioactivity in the reaction was increased to increase the specific activity of the product. An extra 4 uCi of p^*T was added to the stock solution of 40 mM pT in 0.100 M NaOAc (pH 5.0). The addition of an extra 4 uCi of p^*T increased the counts found in the stock solution from around 8,000 to almost 20,500 counts.

After increasing the specific activity of the substrate, an assay was performed using 1500 uM 3'-dGMP as the donor and nucleotide phosphotransfrase concentrations of 30, 40, 50 and 60 ug/ml. This assay was performed twice and the results of both assays showed no increase in product as the concentration of enzyme was increased. Despite the increase in enzyme concentration, the amount of product stayed the same, reaching a maximum of 11% with 60 ug/ml enzyme. The anticipated result was that increasing the enzyme concentration would increase the amount of product. This was the result of my earlier studies with nucleotide phosphotransferase (Figure 2). To confirm the latter result, I perfotmed the assay again with freshly-made 3'-dGMP and pNØp as donors at the different enzyme concentrations. This would confirm that the donors were not responsible for the failure to form increased amounts of product. The fresh solutions did not change the amount of product formed. I also confirmed the concentration of pT in the stock solution by spectrophotometry. Chromatography of

the stock solution by development on a PEI-300 TLC plate with 0.8 M LiCl revealed that only p^*T was present in the stock.

The assays again using 60 ug/ml of enzyme were again repeated. At this point, I noticed that the scans were showing three distinct peaks rather than the two that had been observed previously (Figure 6). Knowing that the labeled carbon originated in the thymidine moiety of the p^*T, I suspected that the new peak was thymidine but looking at the TLC plate under ultraviolet light showed only one spot with R_f of pT. But as Figure 6 showe, there are three distinct radioactive peaks present in this reaction. This result was observed several times with both 3'-dGMP and pN0p as donors. A possible explanation for the lack of a third peak was that the concentration of the nucleotide was too low for it to be visualized. This was suspected because in earlier studies no product would be seen under UV but a scan would reveal product with an R_f corresponding to the known pTp standard. The appearance of thymidine suggested there was a phosphatase present in the reaction mixture and this phosphatase was competing with nucleotide phosphotransferase. In my next experiment, I switched the source of enzyme. I used another concentrated solution of nucleotide phosphotransferase but this change also did not affect the effects. The appearance of the third was still

observed and at the same level as before. Knowing that there was a distinct third peak and suspecting that the third peak was thymidine, I decided to investigate the possible phosphatase activity that was producing the thymidine. A phosphatase is an enzyme that removes phosphate from phosphate containing compounds. In our case, it would cause the formation of *Tp from p*Tp and *T from p*T. A phosphatase can work either on the 3' or 5' phosphate or on both phosphates. I next investigated various enzyme concentrations to see what effect enzyme concentration has on the phosphatase activity. This experiment showed that decreasing the enzyme concentration decreased the phosphatase activity. Also, I added 5 and 10 mM KH_2PO_4 to some of the assays because many phosphatases are inhibited by their product. This experiment showed that decreasing the enzyme concentration decreased the phosphatase activity. The addition of 5 and 10 mM KH_2PO_4 did not inhibit the phosphatase as expected but actually seemed to stimulate the formation of thymidine. The addition of 10 mM KH_2PO_4 increased the amount of thymidine formed in 15 minutes of reaction from 5.0% to 13.3%. The contradiction was that the data from the study of the linear range showed no phosphatase activity whatsoever, even at high enzyme concentrations incubated at 37°C for up to 60 minutes. Phosphatase activity was now present at lower

enzyme concentrations in only 15 minutes of reaction.

To continue my attempts to resolve the contradictory findings, I remade all the reagents. This included new NaOAc buffer, 3'-dGMP and pNØp and p*T solutions. I continued to 60 ug/ml as the concentration for nucleotide phosphotransferase. The result of this experiment again showed phosphatase activity. Its activity was such that after 60 minutes of incubation at 37°C, the third peak accounted for 30% of the radioactivity in the assay while the product accounted for less than 2% of the radioactivity. This TLC was developed on film for 72 hours at -70°C to confirm that the third peak was a separate peak not smeared pT peak. The film confirmed that there was a distinct third peak migrating with an R_f of thymidine.

I then repeated the assays as done in the experiments determining the linear range, where no phosphatase activity was seen. For this experiment, pNØp at 200 mM was the donor, the nucleotide phosphetransferase concentration was 60 ug/ml and the concentration of the acceptor, pT, was 16.67 mM. The results of this experiment are displayed in Figures 7 and 8. Figures 7 and 8 show a large product peak after 15 minutes of reaction but a much smaller product peak after 60 minutes of reaction. Neither time point shows any thymidine. The phosphatase activity was still present. This is shown by the decrease in product with increasing

reaction time. The phosphatase will cleave p*Tp leaving *Tp. Unfortunately, *Tp and p*T comigrate on the TLC sheet and we are unable to tell what forms are present in pT peak. The extent of the phosphatase can be seen by looking at the data from this experiment. After 15 minutes of reaction, 74% of the radioactivity was present as p*Tp, the product of nucleotide phosphotransferase. After 60 minutes of reaction, 44% of the radioactivity was present in the form of p*Tp with the remainder as p*T or T*p. The phosphatase was responsible for this decrease in product by removing the 5'-phosphate from p*Tp. I then tried various donor and enzyme concentrations. I tried pNPp at 15 mM, enzyme at 60 ug/ml; 3'-dGMP at 5.0 mM, enzyme at 45 ug/ml. The results of this experiment showed that by lowering the enzyme concentration to 45 ug/ml, the activity of the phosphatase was greatly reduced and the amount of product, pTp, started to approach the values I had obtained from earlier experiments. I examined the preparation of nucleotide phosphotransferase in a 15% SDS gel to compare it to the enxyme used in the past. A picture of the SDS gel is presented in Figure 9. Lane 1 is the nucleotide phosphotransferase that was used in the earlier study. Lane 2 is the pre-affinity column fraction from the enzyme purification used in the later studies. Lane 3 is the concentrated solution of enzyme from the same

purification. This latter solution is the source for all other enzyme solutions on the gel. Lane 4 is a one to three dilution of lane 3. Lane 5 is a one to two dilution of Lane 4. Lane 5 is the enzyme at the dilution used in the latest experiments. Lane 6 contains markers. Lanes 4 and 5 have a band in them that is not seen in Lane 3. This is unexplainable since the protein in lane 3 is the source for lanes 4 and 5. Also, the proteins in lanes 3, 4, 5 all exhibited phosphatase activity when I tested them. Lane 1 is the enzyme solution from the linear range study that never exhibited any phosphatase activity. Unfortunately, I only had enough enzyme to run the gel and not enough to use in assays. When comparing lane 1 to lanes 2, 3, 4 there is a band in lanes 2, 3, 4 that is not present in lane 1. This may be the band that is causing the phosphatase activity but this remains to be established.

The effects on 10 mM KH_2PO_4 on the phosphatase activity were also examined in this experiment. The addition of 10 mM KH_2PO_4 to a solution containing 20 mM p^*T and 45 ug/ml nucleotide phosphotransferase doubled the activity of the phosphatase an judged by the increase in the amount of thymidine formed. For the solution with no KH_2PO_4 added, the amount of thymidine produced after 15 minutes reaction was 11.9% while a solution with 10 mM KH_2PO_4 added had 23.5% thymidine produced after 15 minutes of reaction. The effect

of KH_2PO_4 on the reaction will be investigated further.

The next experiment investigated the effect KH_2PO_4 had on the phosphotransferase reaction. Assays of 3'-dGMP solutions in the presence of different concentrations of KH_2PO_4 and comparing these to solutions in which no donor was present. The control solutions had 13.33 mMpT, 45 ug/ml nucleotide phosphotransferase and various concentrations of KH_2PO_4. It was expected that some product would be seen in the reactions with no donor because pT can itself function as a donor when no other donor is present. When pT acts a donor, thymidine is produced and this must be considered when determining the amount of thymidine produced by the phosphatase. The results of this experiment are presented in Table 4. A graph of % Thymidine versus concentration of KH_2PO_4 is seen in Figure 10. These results show that in the absence of donor, the phosphatase activity is stimulated. The result that was expected when KH_2PO_4 concentrations were increased was that the phosphatase activity would be inhibited. The result I found, based on the incomplete survey of KH_2PO_4 concentrations, was that the phosphatase activity was not inhibited by increased KH_2PO_4 concentrations but rather the activity was stimulated.

Having found that phosphatase activity could be kept at a minimum when the enzyme concentration was 45 ug/ml, I decided to conduct a final survey of donors with that

concentration for my enzyme. To ensure that the donor was not depleted by the reaction, I brought the donor and acceptor concentrations closer together. For this survey, donor concentration was 5.0 mM and the acceptor, pT, concentration was 6.67 mM. It was hoped this would maximize nucleotide phosphotransferase activity and minimize phosphatase activity. The results of this survey are shown in Tables 5 and 6. The data shows several interesting results. If you compare the donors after 15 minutes of reaction, pN0p is the best donor, producing 182.2% more pTp than does 3'-dGMP but if the donors are compared after 30 minutes of reaction, pN0p produces only 86.7% of the product, pTp, when compared to 3'-dGMP. The donors, compared after 30 minutes of reaction, are listed as normalized to 3'-dGMP from best to worst: 3'-dGMP, pN0p, Acetyl Coenzyme A (l_i + salt), thymidine-3',5'-diphosphate, CoA(Na^+ salt), Acetyl CoA(Na^+ salt), Phosphocreatine, Fructose-1, 6-diphosphate, PEP, 2'-AMP and Phosphatidyl choline. These results are surprising because previous experiments had established 2'-AMP as an excellent donor and my earlier experiments had shown some of the worst donors here had looked were promising. The donor seems to influence the level of phosphatase activity. Donors that led to a high phosphatase activity include PEP, Phosphocreatine and Phosphatidyl choline.

In attempting to find reaction conditions that would allow testing of the lipid derivative dolichol phosphate as a donor, solutions containing various amounts of ethanol were tested. The results of these experiments showed that nucleotide phosphotransfrase were inhibited by ethanol, but that phosphatase was active in solutions containing up to 40% (v/w) ethanol. At 40% ethanol, the phosphatase activity was greatly reduced compared to lower percentages of ethanol but activity was still present. In 10% ethanol solutions, the extent of thymidine after 15 minutes of reaction was 6.4%. In addition, the phosphatase was also active in 40% DMSO, whereas nucleotide phosphotransferase was not. Because of my inability to find a solution where dolichol phosphate was soluble and nucleotide phosphotransferase active, I have been unable to determine if dolichol phosphate would function as a donor.

Discussion

The results of initial studies show the reaction is linear with respect to enzyme concentration and that the assay is reproducable. The reaction was linear with 10 ug/ml to 40 ug/ml of nucleotide phosphotransferase. Using enzyme concentrations within this range, the survey of donors was performed. Based on the results of the final donor survey (Tables 5 and 6), the relationship between

donor and acceptor concentration is extremely important. Because the acceptor, pT, can also act as a donor when another donor is not present, the surveyed donor concentration must be high relative to the pT concentration to ensure that nucleotide phosphotransferase is primarily using the test donor as its phosphate source. One must also be aware that the enzyme can use pT if the test donor is consumed.

If the test donor is consumed or the acceptor concentration is too high relative to the donor concentration, the reaction will then be:

$$p^{*}T \quad + \quad p^{*}T \longrightarrow p^{*}Tp + {}^{*}T$$

(donor) (acceptor)

This reaction will produce $^{*}T$ as shown and this result could be interpreted as being due to a phosphatase when there is not. This possibility had to be considered when I was analyzing my data but was insufficient to explain all of the thymidine produced. In the final donor survey, where the donor concentration was 5.0 mM and the acceptor concentration was 6.66 mM, the amount of thymidine produced is too great to be explained by pT acting as a donor. The reactions in the final donor survey had almost equal donor and acceptor concentrations, yet there was thymidine peaks

with 45% of the radioactivity while the pTp peak contains only 2.5% of the radioactivity. This data supports the possibility that something else besides nucleotide phosphotransferase is present. If this is a phosphatase as suspected, the expected result for the final donor survey, based on equal acceptor concentrations for almost all donors, would be the same phosphatase activity in all assays. Since the phosphatase acts primarily on the acceptor, pT, it would be reasonable to assume that amount of phosphatase activity would not depend on the nature of the donor. The results of the final donor survey show an unexpected result, the activity of the phosphatase appears to depend on the nature of the donor. Donors such as 3'-dGMP, pNØp, Acetyl CuA (Li^+ and Na^+ salt), CoA(Na^+ salt) and thymidine-3', 5'-diphosphate all showed a high level of activity with nucleotide phosphotransferase while the donors 2'-AMP, phoshatidyl choline, PEP and Phosphocreatine all showed a high level of activity with the phosphatase. This was surprising because these donors had been previously identified as good donors for nucleotide phosphotransferase. Since all donors were freshly made up in the NaOAc buffer, results should not reflect any degradation by the acidic buffer. The only donor that showed no observable phosphatase activity, either formation of thymidine or degradation of product was 3'-dGMP. All

other donors showed some degree of phosphatase activity. The data for pNOp are especially striking. In the first 15 minutes of reaction there is a high level of nucleotide phosphotransferase activity, resulting in the formation of 45.0% product with only 0.5% T. In the next 15 minutes, the product decreases suggesting a high level of phosphatase activity resulting in only 23.5% product after 30 minutes. The amount of T stays constant over the reaction, suggesting that only the p is removed from the product pTp. This degradation of product after the first minutes of reaction was also seen in assays using Acetyl CoA(Li^+ and Na^+ salt), CoA(Na^+ salt) and thymidine-3',5'-diphosphate. This result of the nature of the donor affecting the phosphatase activity was not expected just as the result of addition of KH_2PO_4 to the reaction.

I expected that the addition of KH_2PO_4 might inhibit the reaction since many phosphatases are inhibited by their product P_i. The phosphatase activity was in fact not inhibited by the addition of KH_2PO_4 but instead as the concentration of KH_2PO_4 was increased, the phosphatase activity increased. Due to a lack of time, I was unable to perform an assay with sodium arsenate added. Arsenate interferes with the phosphatase by occupying the phosphate site and forming an unstable comound which is immediately hydrolyzed. Determining whether inactivating the

phosphatase also inactivates the phosphotransferase activity might show if the two enzyme activities are part of one enzyme or separate enzymes. If inactivating the phosphatase also inactivates the phosphotransferase, it might suggest the activities are part of one enzyme molecule. If inactivating the phosphatase does not inhibit the phosphotransferase, it might suggest the two enzymes are separate. Also surprising, was the fact that the phosphatase was active in organic solvents that inhibited nucleotide phosphotransferase activity.

Brunngraber and Chargaff noted the phosphatase activity of nucleotide phosphotransferase (12). Their comment on the phosphatase activity was that in the presence of a suitable acceptor the phosphatase activity of the transferase is greatly, if indeed not completely, inhibited. They do not report any phosphatase activity when a suitable donor and acceptor are present, like I have observed.

To extend this investigation, I would propose to investigate the effects of KH_2PO_4 on the phosphatase activity, perform assays with sodium arsenate and to investigate the donor-acceptor relationship on phosphatase activity. I would also confirm the phosphatase results by redoing these experiments on the next enzyme purification and see if the results are reproducable. If the results are consistent from purification to purification, the

phosphatase activity might help explain the biological function of this enzyme by providing clues to possible functions a combined phosphotransferase-phosphatase enzyme might have.

Table 1

RESULTS OF LINEAR RANGE EXPERIMENTS

Reaction time (minutes)

	0	3	7	13	20	30
40	16.4	49.8	79.3	76.2	79.2	80.5
30	14.8	35.0	43.1	56.6	74.6	76.0
20	12.6	21.8	32.4	46.2	65.7	67.8
10	21.5	23.5	26.0	34.3	38.9	45.3
5	10.0	19.3	21.0	22.5	23.9	27.6

TABLE 1 % pTp PRODUCED IN LINEAR RANGE EXPERIMENTS

Reactions were performed as described in Methods section.

Table 2

REACTION VELOCITY OF LINEAR RANGE EXPERIMENTS

enzyme concentration	% pTp produced per minute
40	7.4
20	4.1
20	2.7
10	0.8
5	0.6

TABLE 2 REACTION VELOCITY OF LINEAR RANGE EXPERIMENTS

The table shows the % pTp produced/minute for the indicated enzyme concentrations. The experiments were performed as described in Methods section.

TABLE 3

RESULTS OF FIRST DONOR SURVEY

donor	3'-dGMP			2'-AMP			pNØp			PEP			Phosphitylcholine			Phosphocreatine		
uM	1500			1500			1500			1500			1500			1500		
rxn time	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30
% pTp	3.0	6.8	8.1	3.5	6.0	7.8	2.7	4.8	5.8	2.8	6.1	7.1	4.3	5.5	7.1	2.8	5.7	7.1
pTp produced as %product with 3'-dGMP as donor	-	-	-	117	88	96	90	71	79	93	90	88	143	81	88	93	84	88
uM	500			500			500			500			500			500		
% pTp	3.1	5.3	7.8	4.2	4.6	6.6	3.9	4.8	6.7	3.2	5.4	6.6	4.3	6.7	6.6	3.9	5.8	7.4
pTp produced as %product with 3'-dGMP as donor	-	-	-	135	87	85	125	91	86	103	102	85	138	126	85	126	109	95
uM	100			100			100			100			100			100		
rxn time	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30
% pTp	3.0	5.6	7.0	3.6	6.2	6.6	4.4	5.0	5.2	3.9	4.9	8.4	6.3	6.6	9.4	3.4	4.6	6.5
pTp produced as %product with 3'-dGMP as donor	-	-	-	120	111	94	147	89	75	130	88	120	210	118	135	113	82	93

TABLE 3 RESULTS OF FIRST DONOR SURVEY

Each donor surveyed is compared to the 3'dGMP Standard in the amount of p*Tp produced.

Table 3 (continued)

Results of First Donor Survey

donor	3'-dGMP			thymidine 3',5' diphosphate			Acetyl Coenzyme A Li+ salt			Fructose-1,6-diphosphate		
mM	1500			1500			1500			1500		
rxn time	0	15	60	0	15	60	0	15	60	0	15	60
% pTp	3.0	6.8	8.1	3.5	6.5	6.8	3.3	5.1	6.1	3.9	5.0	6.1
pTp produced as %product with 3'-dGMP as donor	-	-	-	117	96	78	110	75	75	130	74	75
mM	500			500			500			500		
% pTp	3.1	5.3	7.8	4.6	4.7	7.4	4.3	5.8	7.5	4.1	4.1	5.9
pTp produced as %product with 3'-dGMP as donor	-	-	-	146	89	95	138	109	96	132	77	76
mM	100			100			100			100		
rxn time	0	15	60	0	15	60	0	15	60	0	15	60
% pTp	3.0	5.6	7.0	3.9	6.9	7.8	5.3	5.6	7.1	4.2	5.7	7.8
pTp produced as %product with 3'-dGMP as donor	-	-	-	130	123	115	176	100	101	140	102	111

TABLE 4

	Rxn Time	0	15	30
	3'-dGMP 3.75mM	1.2	1.8	1.5
	control (no donor)	0.7	2.4	2.3
w/10mM KH2PO4	3'-dGMP 3.75mM	0.6	1.2	1.1
w/10mM KH2PO4	control (no donor)	1.6	12.9	23.5
w/20mM KH2PO4	3'-dGMP 3.75mM	1.0	0.6	2.0
w/20mM KH2PO4	control (no donor)	13.4	18.1	32.9
w/30mM KH2PO4	3'-dGMP 3.75mM	2.2	5.9	1.9
w/30mM KH2PO4	control (no donor)	1.9	17.2	23.6
w/40mM KH2PO4	3'-dGMP 3.75mM	4.3	4.7	3.8
w/40mM KH2PO4	control (no donor)	5.0	19.2	20.7
w/50mM KH2PO4	3'-dGMP 3.75mM	7.0	1.3	2.7
w/50mM KH2PO4	control (no donor)	5.3	20.9	33.3
w/250mM KH2PO4	3'-dGMP 3.75mM	5.7	2.7	4.5
w/250mM KH2PO4	control (no donor)	5.4	11.9	19.9

% Thymidine produced

TABLE 4 RESULTS OF VARYING KH_2PO_4 ON AMOUNT OF THYMIDINE PRODUCED

Reactions were performed with 3.75 mM 3'-dGMP as the donor, 13.33 mM pT as the acceptor and 45 ug/ml nucleotide phosphotransferase. The controls are the same except 10 ml of NaoAc buffer instead of 3'-dGMP.

Table 5

Donor	donor conc. (mM)	rxn time (minutes)	%pTp	%pT	%T
3'-dGMP	5.0	0	1.7	94.9	0.8
"	"	15	24.7	73.4	0.5
"	"	30	27.1	70.7	0.5
pNPp	5.0	0	4.6	93.2	0.5
"	"	15	45.0	52.5	1.0
"	"	30	23.5	72.5	2.3
3'-AMP	2.5	0	2.1	95.9	0.4
"	"	15	3.2	87.9	7.0
"	"	30	2.4	78.7	17.3
[illegible]	5.0	0	2.2	96.4	0.8
"	"	15	3.0	86.1	10.3
"	"	30	2.0	78.3	17.7
PEP	"	0	2.1	92.1	4.1
"	"	15	3.9	65.8	30.4
"	"	30	2.5	49.6	45.6
[illegible]	"	0	1.9	96.4	6.3
"	"	15	4.8	78.2	17.1
"	"	30	4.5	68.6	27.2
AcCoA,Na+Salt	"	0	2.3	76.8	18.8
"	"	15	11.8	70.4	15.9
"	"	30	8.4	79.9	9.7
AcCoA,Li+Salt	"	0	2.1	92.1	3.4
"	"	15	14.7	79.2	3.7
"	"	30	12.5	77.1	8.0
CoA,Na+Salt	"	0	2.2	95.3	2.1
"	"	15	12.0	85.5	2.6
"	"	30	10.4	85.8	3.8
[illegible]	"	0	3.1	92.5	0.4
"	"	15	19.9	78.1	1.2
"	"	30	11.2	88.1	4.0
[illegible]	"	0	3.5	94.0	1.1
"	"	15	4.0	90.5	3.6
"	"	30	2.9	88.1	9.1
[illegible]	-	0	1.5	93.1	4.0
"	-	15	4.5	90.9	3.1
"	-	30	3.2	90.5	6.1

Table 5 RESULTS OF DONOR SURVEY

Nucleotide phosphotransferase concentration was 45 ug/ml. All reactions were incubated at 37°C

TABLE 6

DONOR SURVEY RESULTS

donor	3'-GMP			PNPP			AcCoA, Na+Salt			AcCoA, Li+Salt			CoA,Na+ Salt			Thymidine-3',5'-diphosphate		
donor conc. (mM)	5.0			5.0			5.0			5.0			5.0			5.0		
rxn time (minutes)	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30
% pNp produced	1.7	24.7	27.1	4.6	40.0	22.5	2.3	11.8	8.4	2.1	14.7	12.5	2.2	12.0	10.4	3.1	19.0	11.2
pNp product as % produced with 3'-GMP as donor	-	-	-	276.6	102.2	83.7	135.3	47.8	36.9	123.5	59.5	46.1	129.4	48.6	36.3	102.3	78.9	41.3
donor	Phosphoenol pyruvate			PEP			Phosphocreatine			Fructose-1,6-diphosphate			2'-AMP			control (no donor)		
donor conc. (mM)	5.0			5.0			5.0			5.0			2.5			-		
rxn time (minutes)	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30
% pNp produced	2.2	3.0	2.0	2.1	3.9	2.5	1.9	4.6	4.5	4.0	2.0	1.0	2.1	3.2	2.4	1.0	4.6	3.2
pNp product as % produced with 3'-GMP as donor	129.4	12.2	7.3	128.5	25.6	9.2	111.7	10.0	10.0	206.3	11.7	6.0	123.5	12.0	8.0	94.1	10.2	11.0

TABLE 6 SURVEY OF DONORS

All reactions had a nucleotide phosphotransferase concentration of 45 ug/ml. Reactions were performed at 37° C for the indicated time.

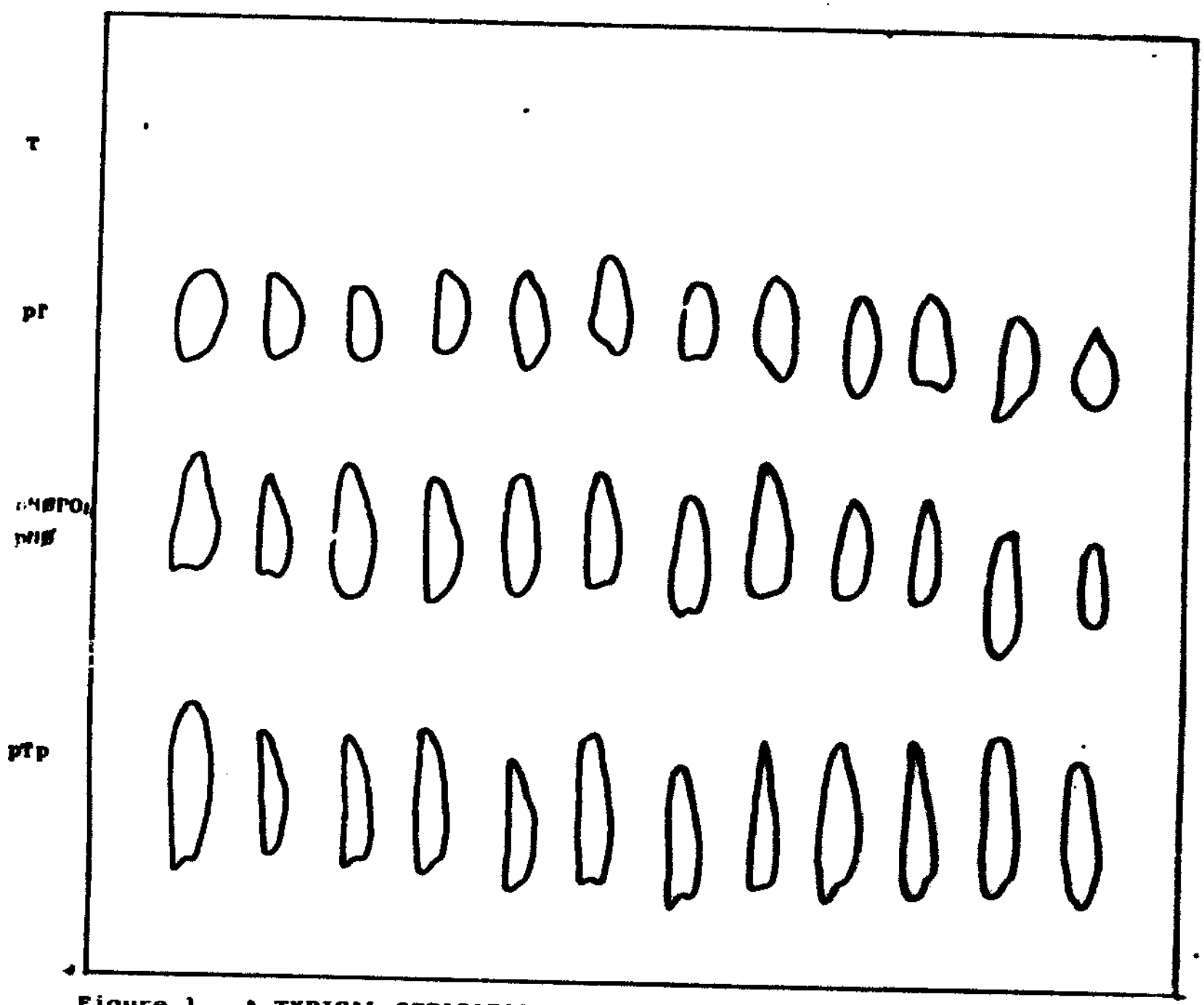


Figure 1. **A TYPICAL SEPARATION OF PRODUCTS IN AN ASSAY REACTION**
The patterns shown were obtained from a developed PEI-300 chromatogram while visualizing spots under ultraviolet illumination. The plate was developed at 4.0°C with 0.8 M LiCl.

Figure 2 LINEAR RANGE RESULTS

Reactions were run with the conditions as listed in the methods section for the linear range assay.

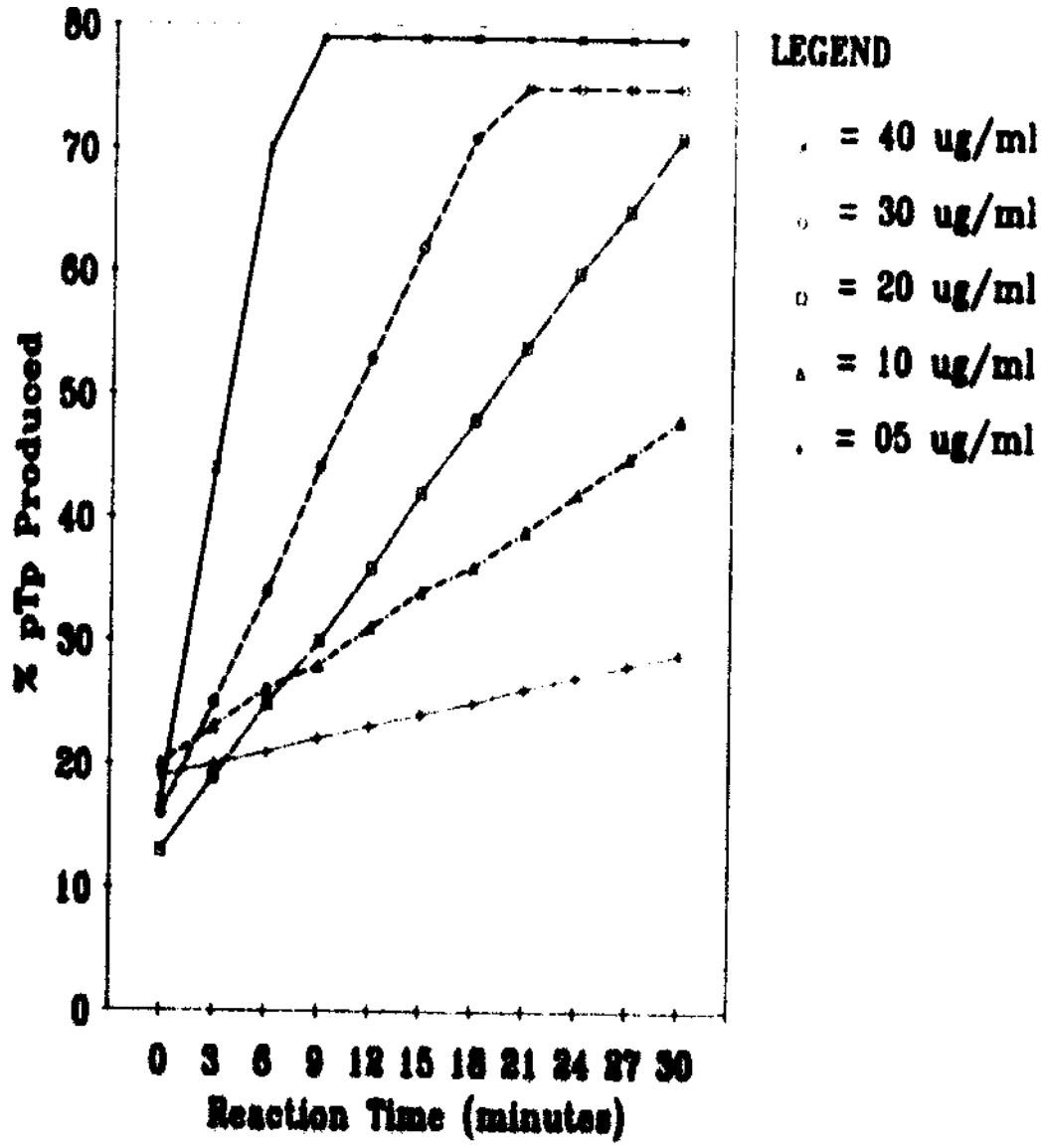
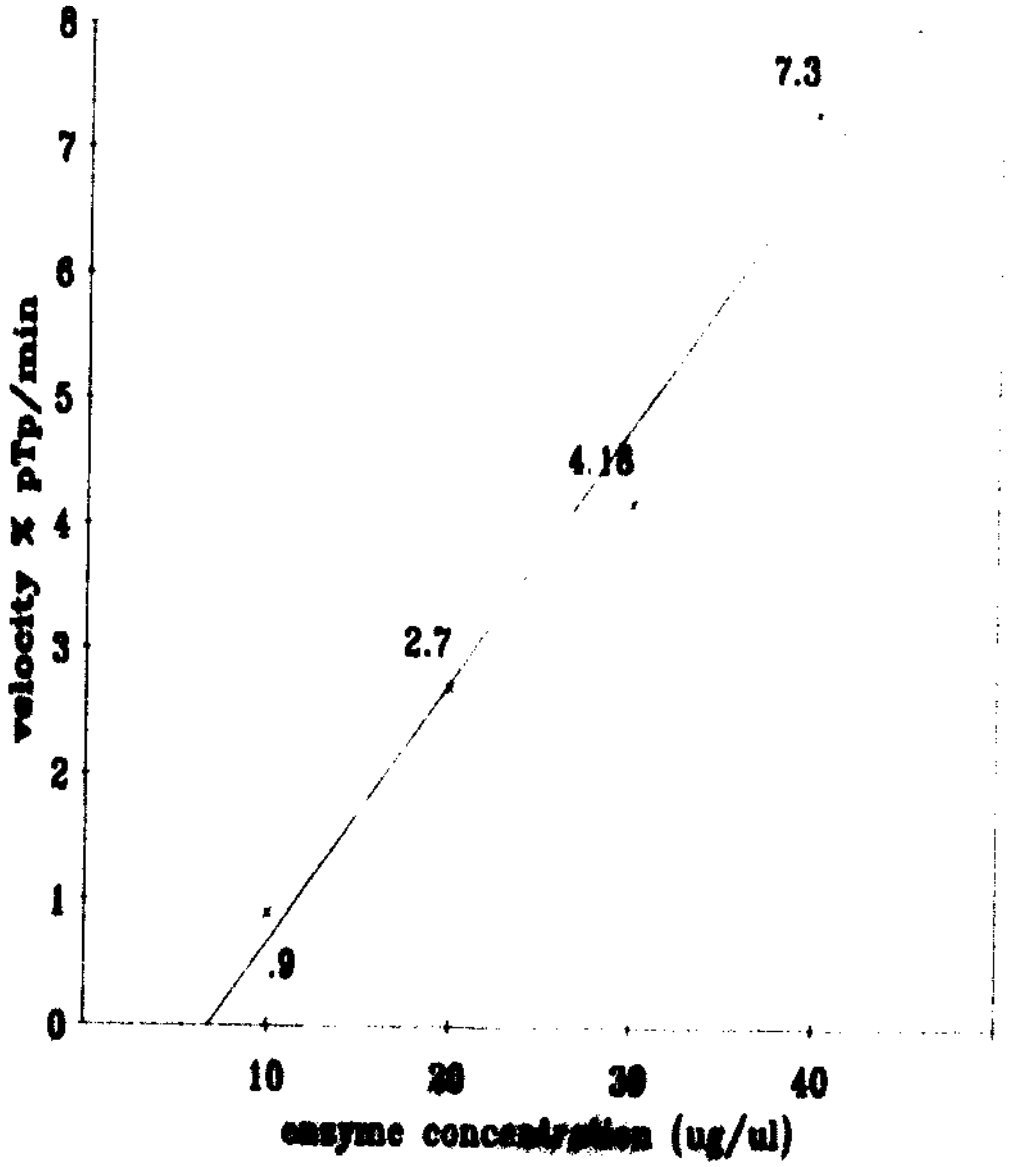


Figure 3 LINEAR RANGE FOR REACTION VELOCITY
Reactions were run with the conditions as listed in the methods section for the linear range assay.



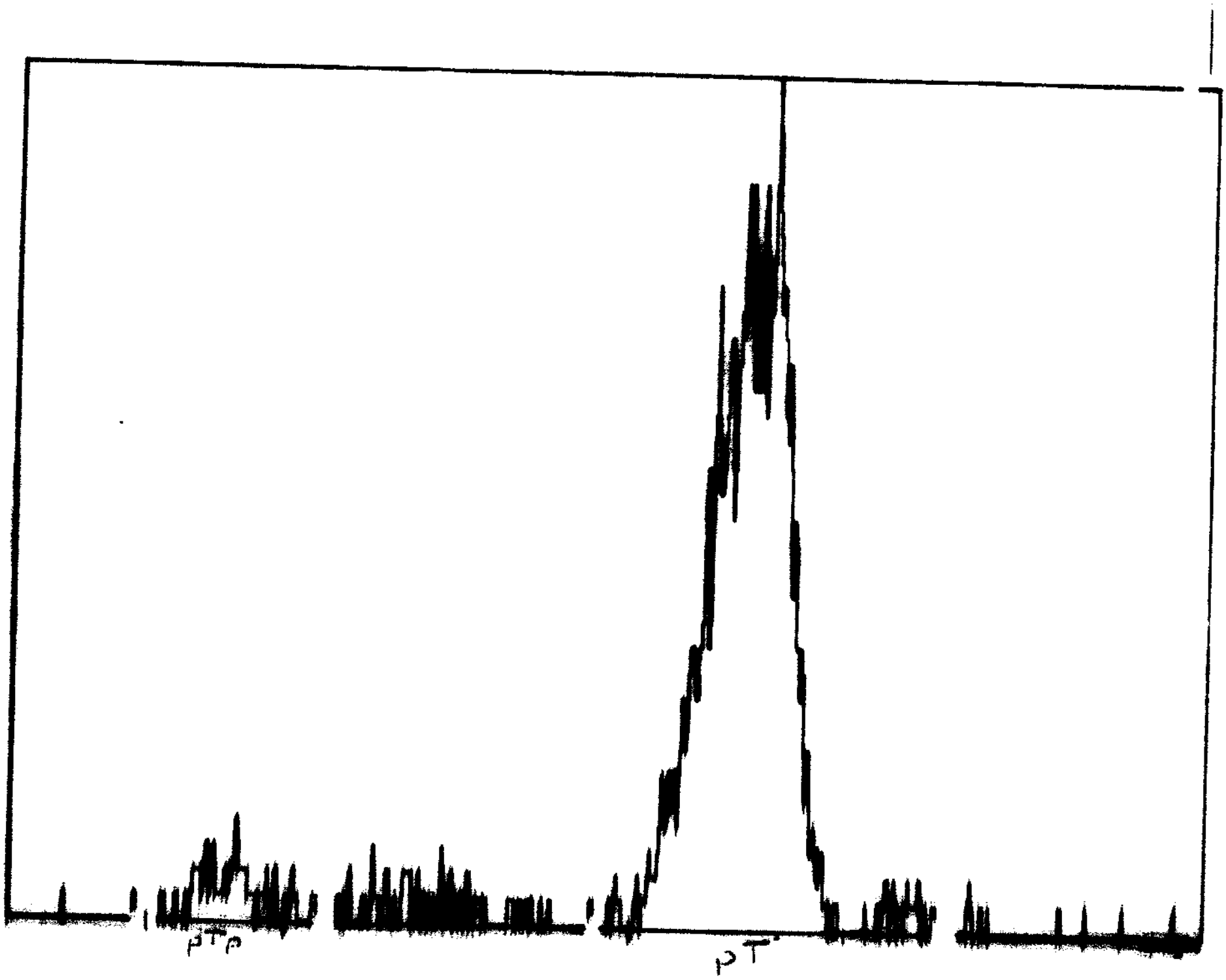


Figure 4. SCAN OF A DONOR SURVEY USING 3'-dGMP AS THE DONOR.
Reaction conditions were 500 uM 3'-dGMP as the donor and 13.33 mM pT as the acceptor. The reaction was incubated at [illegible]

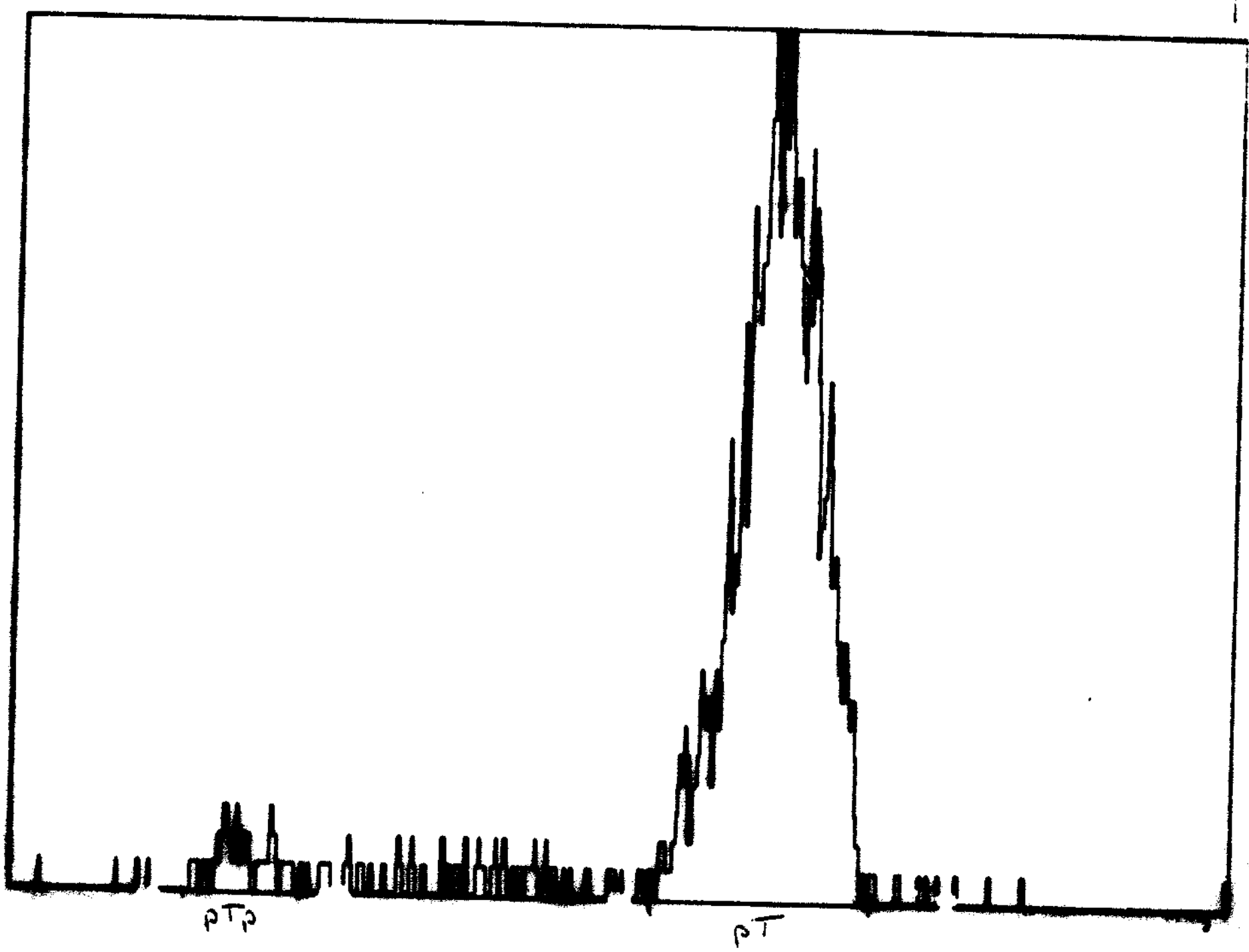


Figure 5. SCAN OF A DONOR SURVEY ASSAY USING THYMIDINE-3',5'-DIPHOSPHATE AS THE DONOR.
Reaction conditions were 1500 uM thymidine-3',5'-

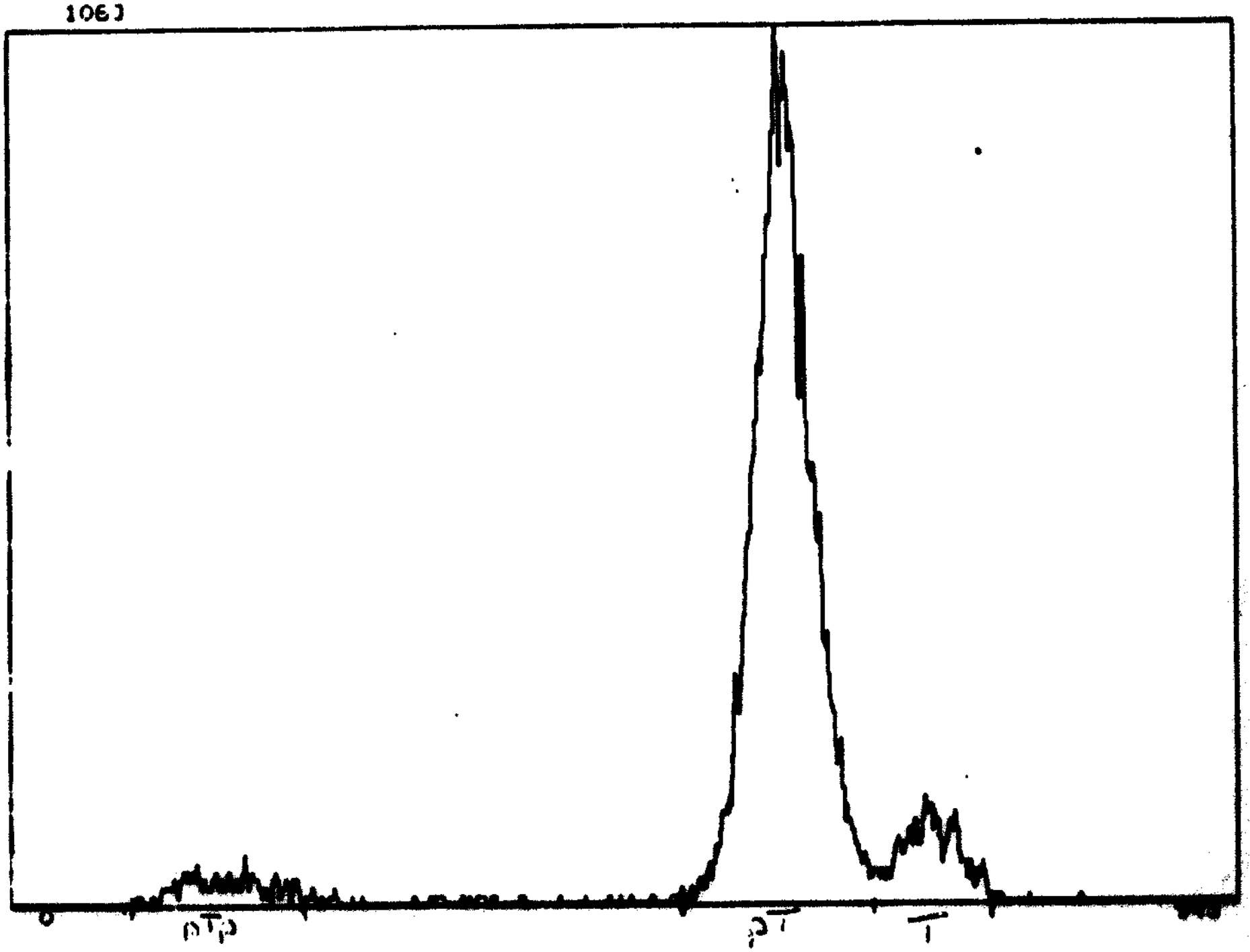


Figure 6. SCAN OF A DONOR SURVEY USING 3'-dGMP AS THE DONOR Reaction conditions were 500 uM 3'-dGMP as the donor and 16.67 mM pT as the acceptor. The reaction was incubated at 37°C for 15 minutes before being stopped with 1M KOH. Nucleotide phosphotransferase concentration was 60 ug/ml.

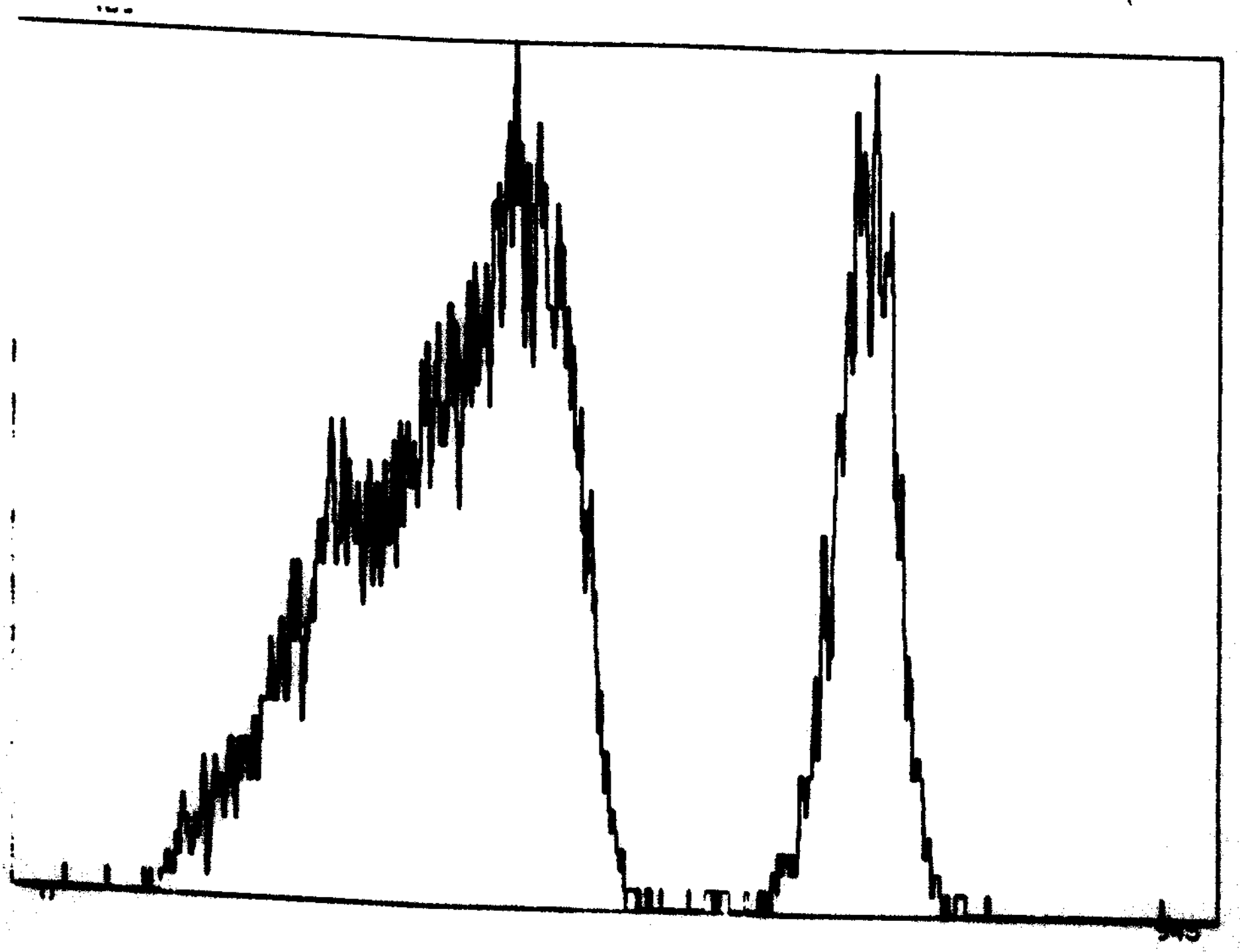


Figure 7. SCAN OF A DONOR ASSAY USING pNOp.
Reaction conditions were 200 mM pNOp as the donor and 16.67 mM pT as the acceptor. Nucleotide phosphotransferase concentration was 60 ug/ml. The reaction was incubated at 37°C for 15 minutes before being stopped with 1N KOH.

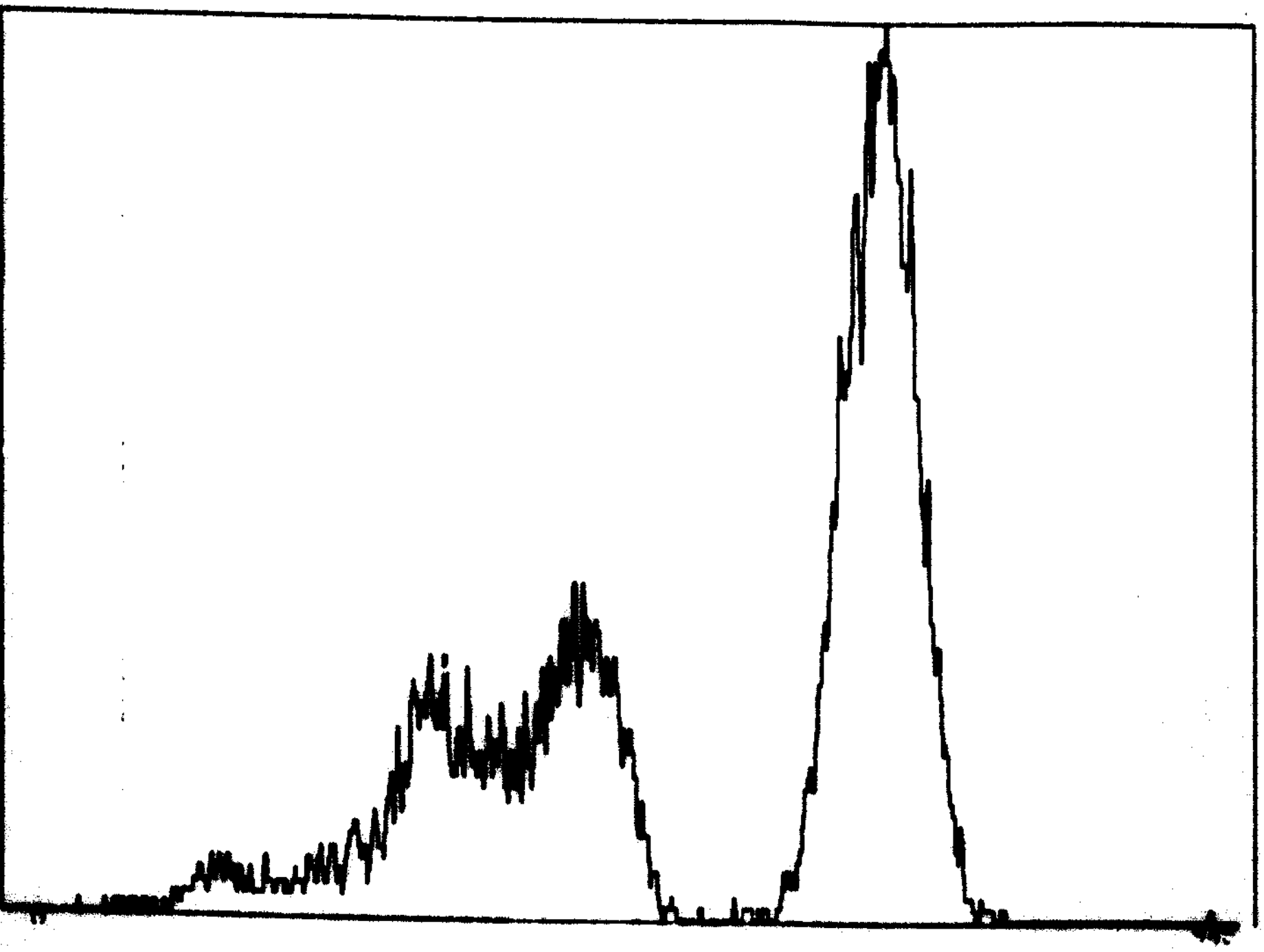
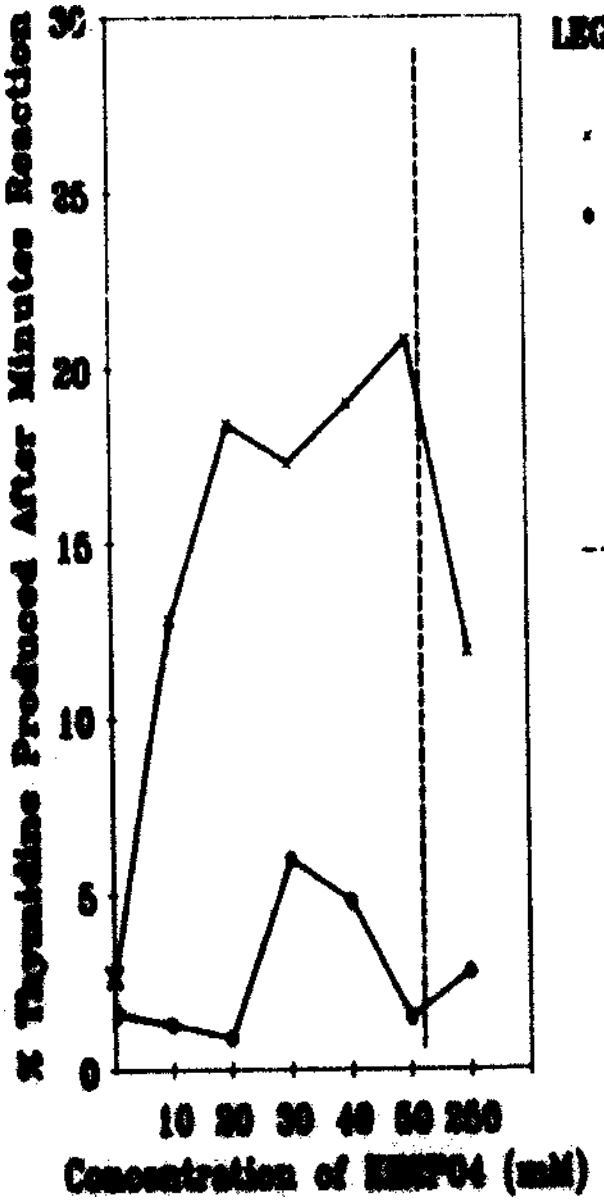


Figure 8. SCAN OF A DONOR ASSAY USING pNØp
Reaction conditions were 200 mM pNOp as the donor and 16.67 mM pT as the acceptor. Nucleotide phosphotransferase concentration was 60 ~g/mℓ . The reaction was incubated at 37°C for 60 minutes before being stopped with 1N KOH.

1 2 3 4 5 6

Figure 9. 15 SDS GEL

Figure 10-X THYMIDINE PRODUCED
VERSUS
CONCENTRATION OF KH2PO4



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