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(Plant Bioenergetics)
Project Reports (2)

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THE INSTITUTE OF PAPER CHEMISTRY

QUARTERLY RESEARCH REPORT

DIVISION: Natural Materials & Systems

DATE: October 20, 1975

✓ PROJECT NO: 2691, Mechanisms in Bioenergetics of Plants

Project Leader: Morris Johnson

Class: FE

OBJECTIVE: Overall - gain a more complete understanding of the biochemical mechanisms whereby green plants trap, store and utilize energy released during biological electron transport. Specific - isolate and characterize the apparently reversible adenosinetriphosphatase (ATPase) of cabbage mitochondria.

BUDGET: \$15,000 per year

SCHEDULE: Project is on hold at least until July, 1975

SUMMARY OF RESULTS AND PLANS FOR FUTURE WORK: No laboratory work was conducted this quarter. This project remains on hold pending a review of a plan of action embodied in my memorandum to John Seanson dated December 19, 1974. That memorandum reviews the current status of the project and provides two alternatives in the event the project resumes.

Report Prepared by Morris Johnson

THE INSTITUTE OF PAPER CHEMISTRY

QUARTERLY RESEARCH REPORT

DIVISION: Natural Materials & Systems

DATE: July 7, 1975

PROJECT NO.: 2691, Mechanisms in Bioenergetics of Plants

Project Leader: Morris Johnson

Class: FE

OBJECTIVE: Overall - gain a more complete understanding of the biochemical mechanisms whereby green plants trap, store and utilize energy released during biological electron transport. Specific - isolate and characterize the apparently reversible adenosinetriphosphatase (ATPase) of cabbage mitochondria.

BUDGET: \$15,000 per year

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Report Prepared by Morris Johnson

THE INSTITUTE OF PAPER CHEMISTRY

QUARTERLY RESEARCH REPORT

DIVISION: Natural Materials & Systems

DATE: March 31, 1975

✓ PROJECT NO.: 2691, Mechanisms in Bioenergetics of Plants

Project Leader: Morris Johnson

Class: FE

OBJECTIVE: Overall - gain a more complete understanding of the biochemical mechanisms whereby green plants trap, store and utilize energy released during biological electron transport. Specific - isolate and characterize the apparently reversible adenosinetriphosphatase (ATPase) of cabbage mitochondria.

BUDGET: \$15,000 per year

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Report Prepared by Morris Johnson

THE INSTITUTE OF PAPER CHEMISTRY

QUARTERLY RESEARCH REPORT

DIVISION: Natural Materials & Systems

DATE: January 15, 1975

✓ PROJECT NO.: 2691, Mechanisms in Bioenergetics of Plants.

Project Leader: Morris Johnson

Class: FE

OBJECTIVE: Overall - gain a more complete understanding of the biochemical mechanisms whereby green plants trap, store and utilize energy released during biological electron transport. Specific - isolate and characterize the apparently reversible adenosinetriphosphatase (ATPase) of cabbage mitochondria.

BUDGET: \$15,000 per year

SCHEDULE: Project is on hold at least until July, 1975

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Report Prepared by Morris Johnson

THE INSTITUTE OF PAPER CHEMISTRY

QUARTERLY RESEARCH REPORT

DIVISION: Natural Materials & Systems

DATE: October 1, 1974

PROJECT NO.: 2691, Mechanisms in Bioenergetics

Project Leader: Morris Johnson

Class: FE

OBJECTIVE: Overall - gain a more complete understanding of the biochemical mechanisms whereby green plants trap, store and utilize energy released during biological electron transport. Specific - isolate and characterize the apparently reversible adenosinetriphosphatase (ATPase) of cabbage mitochondria.

BUDGET: \$15,000 per year

SCHEDULE: Project is on hold until the Protoplast Fusion research (Project 3223) is on a firm footing. Discussion with Mr. Posner indicates that federal funding for this project may be sought in the future.

SUMMARY OF RESULTS AND PLANS FOR FUTURE WORK:

Only a small amount of research has been conducted since the date of the last project review (February 8, 1974) due to precedence of Project 3223. In this work some variations of both cation and anion exchange chromatography were employed and the fractions were assayed for ATPase and examined by disc electrophoresis. In the last couple of experiments a rapid on-gel stain for ATPase activity was used which allows one to see which protein bands are active.

PROJECT NO.: 2691

DATE: October 1, 1974

Up to four bands of activity were found (one very weak) to be fractionating on the ion exchange columns. The most active ATPase ion-exchange fractions had active bands much like active ATPase fractions from salt fractionation. It remains to be seen whether ATPase purified by ion exchange still has ATP synthetase activity (i.e.; is reversible).

Electrophoresis is by far the best fractionating system in this work but we have only used it analytically since our preparative apparatus is homemade and has never performed well. Combined with on-gel staining to locate active bands (ATPase) which could be eluted and quantitatively assayed for ATPase and ATP synthetase activity, preparative electrophoresis may be the fastest way to bring the question of ATPase reversibility to a head. If an eluted band or some combination of eluted bands has ATP synthetase activity, the reversibility question could be resolved. Characterization work could then proceed with the isolated band or bands. If an ATP synthetase active preparation is loaded on the preparative electrophoresis column but can't be recovered after electrophoresis as a single band or combination of bands, then the electrophoresis would have destroyed some necessary organization and the method would be incapable of resolving the question without modification. Nevertheless, preparative electrophoresis or some similar procedure like preparative electrofocusing or density gradient electrophoresis will be of high priority when the work resumes.

Plans and objectives for the fourth quarter of 1974 -

No work planned; funding and plans for the project to be reviewed in second quarter of 1975.

Report Prepared by Morris Johnson

THE INSTITUTE OF PAPER CHEMISTRY
QUARTERLY RESEARCH REPORT

DIVISION: Natural Materials & Systems

DATE: October 1, 1974

✓ PROJECT NO.: 2691, Mechanisms in Bioenergetics

Project Leader: Morris Johnson

Class: FE

OBJECTIVE: Overall - gain a more complete understanding of the biochemical mechanisms whereby green plants trap, store and utilize energy released during biological electron transport. Specific - isolate and characterize the apparently reversible adenosinetriphosphatase (ATPase) of cabbage mitochondria.

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Report Prepared by Morris Johnson

PROJECT REPORT FORM

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✓ PROJECT NO. 2691
COOPERATOR Institute of Paper
REPORT NO. Five Chemistry
DATE June 20, 1973
NOTE BOOK 2871 pp. 71-160 & 2957
PAGE 1 TO 116
SIGNED *M. A. Johnson*
Morris A. Johnson

MECHANISMS IN BIOENERGETICS OF PLANTS

SUMMARY

Ammonium sulfate fractionation monitored by enzyme assays and disk electrophoresis shows that ATP synthetase and ATPase fractionate together. Active fractions consist primarily of two nucleoproteins as determined by staining characteristics and chemical analysis. The salt fractionation also resolves the succinic thiokinase contamination. Combined gas and paper chromatographic analyses leave little doubt that G-6-³²P is the product of the intermediate assay. RNase inhibition of G-6-³²P formation is probably real but not by a mechanism of real interest; the more relevant effect may be stimulation. To date there is no evidence that extract is capable of polyadenylic acid phosphorylation.

INTRODUCTION

Sufficient progress was made in this project over the last several months to allow preparation and submission of a manuscript to Biochimica et Biophysica Acta. Its fate is unknown at this time. Further investigation of some items summarized in the last report requires revision of some statements made at that time. For example, the time progress curve for G-6-P formation which shows apparent destruction of G-6-P was thought to be due to enzymes contaminating the hexokinase; it has been found that hexokinase has mutarotase activity which may be a contributing factor, but the mitochondrial extract itself has both mutarotase and glucose 6-phosphatase activity. It was observed previously that RNase was an inhibitor of G-6-P formation from P_i but that puzzling effects occurred when the RNase was added after the reaction had

started. New experiments in which extract was preincubated with RNase before initiating the reaction which incorporates P_i into G-6-P (ATP synthetase) resulted in stimulatory rather than inhibitory effects. There is evidence that the RNase inhibition observed is due to binding of P_i by RNase rather than effects on macromolecules.

Since it was rediscovered that spinach succinic thiokinase (STKase) can't substitute pantetheine for CoA as animal STKases can, the idea that the observed ATP- P_i exchange might not be STKase has fizzled. However, several experiments have indicated that ammonium sulfate fractionation resolves this activity from ATP synthetase, so it is no longer the great source of concern that it once was. Salt fractionation also results in the recovery of transfer and ATPase activity in the same fraction from which not only STKase but G-6-Pase appears to be excluded. By employing analytical disk electrophoresis along with enzyme assays to monitor the salt fractionation, it was found that the active salt fraction mainly consists of the two major bands of low electrophoretic mobility (cf. Report no. 2, Fig. 1) which were found to stain for protein, nucleic acid, and ATPase. A preliminary characterization of RNA from deproteinized extract has resulted in the identification of adenine, uracil, and guanine or their derivatives in the nucleoprotein. Attempts to demonstrate endogenous polynucleotide phosphorylase activity have been negative to date. Gas chromatography with subsequent paper chromatography of collected peaks has shown that the reaction product is G-6- ^{32}P from both P-6 excluded extracts and active salt fractions derived therefrom.

RESULTS AND DISCUSSION

STKase ATP-³²P_i Exchange

At the close of the last report some data were presented which showed that pantetheine did not substitute for CoA in the STKase ATP-³²P_i exchange reaction catalyzed by extract. Some of these data are presented in Table I along with results of an identical type of experiment conducted with whole cabbage mitochondria. It is of interest to contrast these results in terms of what is known about the ATP-P_i exchange of oxidative phosphorylation versus the ATP-P_i exchange of STKase. Note that the extract exchange is completely dependent upon CoA addition while the mitochondrial exchange is not. This could be interpreted that the mitochondria have sufficient CoA or that this exchange doesn't involve CoA. The elimination of succinate shows that the exchange in mitochondria is less dependent on succinate than in extract. The DNP results mean that most of the exchange in mitochondria is not STKase exchange contrary to the exchange of extract. Note also that the exchange in mitochondria is much more susceptible to PCMB than in extract; this correlates well with our earlier report (1) that ATP synthetase is inhibited by PCMB more severely than is STKase. The atractyloside results are difficult to interpret because this inhibitor is supposed to be effective against STKase from animal (plant?) systems. Likewise, there doesn't seem to be any literature on the subject of RNase inhibition of these exchanges. Despite the interpretation made in the last report the failure of pantetheine to substitute for CoA does not indicate that the extract exchange is not STKase; such an interpretation would follow for animal systems but not plant systems (2). The best evaluation at present is that most of the count under the extract column represents STKase exchange while little if any STKase exchange is found under the mitochondria column

regardless of the fact that STKase originates from mitochondria. It seems possible that the value 346 may not represent STKase exchange but rather some CoA-dependent reaction (ATP synthetase?, exchange?) related to oxidative phosphorylation. It will be of interest in the future to look at the effect of DNP and PCMB upon an extract exchange assay lacking succinate, i.e., do they inhibit the value like 346. If they do (particularly if DNP does), there would indeed seem to be a CoA-dependent ATP-P_i exchange present in addition to STKase ATP-P_i exchange. As with all results based upon nonextractable ³²P, in that case, it would be necessary to verify that the involved count is really AT³²P.

Fractionations and Analyses

A few isolated experiments in the past had indicated that STKase could be separated from ATP synthetase by salt fractionation in which only ATP synthetase precipitated at 50% saturation (0° C). As this work was taken up again, some difficulty was experienced in placing salt precipitates back into solution; however, this was due apparently to the grade of ammonium sulfate employed since it no longer has been a problem with ultrapure salt. In addition to the usual assays, it was decided to employ analytical disk electrophoresis to monitor the progress of the fractionation in terms of heterogeneity of the sample macromolecular complement. The electrophoresis also afforded the opportunity to check staining reactions, including one for P_i liberated from ATP (i.e., an on-gel test for ATPase activity).

The reconstitution problem encountered before acquisition of ultrapure ammonium sulfate is illustrated in the first experiment of Table II. In this case the entire extract was first precipitated at 70% saturation. Attempts to

redissolve this material for further fractionation and assay were only partially successful; therefore, only in the 50-57% and 57-70% fractions were the cuts made from clear solutions. No ATP synthetase activity was recovered in any fraction in this run while STKase exchange appeared in the 57-70% cut. Another "normal" fractionation of STKase appears in the same table. Recovery of STKase is always low (more readily apparent if placed on per mg. protein basis) but it is never recovered at low saturation levels; one activity loss factor appears to be lack of dialysis, but it is probably not the full explanation.

The first electrophoresis pattern obtained for a salt fraction compared with crude extract is diagrammed in Fig. 1. It is readily evident that the major components in the 0-50% salt fraction are two bands of low mobility which stain for both protein (a) and nucleic acid (b). Fig. 1 (c) depicts the appearance of 0-45% versus 45-50% fractions and, since little was gained by going to 50% with increasing danger of precipitating some STKase, the saturation level for ATP synthetase isolations was reduced to 45% for routine work. In Fig. 1(d) one can note that there is little salting out below 30% and what does appear is the same material that predominates in 30-40% fractions; therefore ATP synthetase fractions were further standardized to 0-45% salt precipitates. Banding patterns of a 0-45% fraction before and after treatment with pancreatic RNase as well as some pH adjustment-generated fractions (cf. report no. 4) of crude extract are shown in Fig. 2 (a)(b). There was no obvious effect of the pancreatic RNase. Concentrations of the pH precipitates could have been greater, but, most noteworthy is the fact that, although precipitation at pH 5.3 is substantial, considerable proteinacious

material remains in solution; one of these bands still in solution appears to be the major band in 0-45% salt fractions and there is a major band of greater mobility which would seem to have been generated from other components as a result of the pH adjustment! This observation may be of more than passing importance since when coupling factor A (or A.D) of Sanadi's group (3) is purified, the first step is a pH 5.6 precipitation to remove unwanted components. More experiments must be done to ascertain if (1) pH 5.3 supernatants' major bands still stain for nucleic acid (tried here but too dilute to tell) and (2) pH 5.3 supernatants have ATP synthetase capacity. There is some indication already that the answer to (1) is no; consequently, if pH 5.3 supernatant (and other pH fractions) are inactive, it may be possible to show that the reason is that the RNA has been separated from the nucleoprotein. Alternatively if the answer to (1) is no, and pH 5.3 supernatants are active, then RNA is not essential for ATP synthesis. A well-resolved, Coomassie blue-stained, electrophoretic pattern of crude extract and of a 0-45% fraction is shown in Fig. 2 (c). The effect of pre-electrophoresis upon the resolution of a 0-45% salt fraction is seen in Fig. 2(d); although not used routinely, it does appear to offer improved resolution for this preparation. It was possible to show that the major band regions in Fig. 2 (d) stain also for ATPase activity, but it is very difficult to take a good photograph of the fleeting blue color. Also examined was the banding pattern of a 0-45% fraction as affected by preincubation with the hexokinase trap or ADP alone. There were no obvious changes; apparently hexokinase resolves into two subunits. It may be seen that the same salt fraction in Figs. 1 and 2 does not always yield precisely the same banding pattern reflecting concentration effects during salting out and electrophoresis. The behavior of ATP synthetase upon salt fractionation of crude extract is shown in the experiment of Fig. 3 which shows recovery in a 0-45% fraction. Fig. 4

shows an experiment with not only ATP synthetase recovery but also purification in the 0-45% fraction derived from P-6 excluded crude extract. The gas chromatographic analysis of G-6-P also revealed mutarotase and G-6-Pase activity in the extracts (Fig. 5); the former was also found in the hexokinase used. In Fig. 5 the mutarotase results in anomer peaks for G-6-P and the phosphatase activity results in α and β glucose production (on side of solvent front) from G-6-P. These activities were reduced but not completely eliminated in 0-45% fractions and are thought to be responsible for G-6-P disappearance. The distribution of G-6-Pase and ATPase as determined by P_i analysis is shown in Fig. 6. Some characteristics of the ATPase are present in Table III. Two experiments were run (one in Fig. 7) employing ^{14}C glucose rather than $^{32}P_i$ for the ATP synthetase assay. The distribution of activity in salt fractions appears to be the same as when $^{32}P_i$ is used. This may be X~P harvest but it could also be harvest of ATP formed by adenylate kinase action on the ADP provided. (An experiment utilizing ^{14}C ADP to measure adenylate kinase activity still has not been run.) In one of these experiments it was found that the supernatant after precipitation with 75% ammonium sulfate still had a considerable amount of orcinol-positive material in it that could not logically be ascribed to MOPS, acetone, or sucrose (possibly fructose? -- if sucrose hydrolyzed). A sample of this material evaporated at 28° C (eliminates acetone) reconstituted and scanned, showed a peak at 281 nm. (hydroxymethyl furfural?). Although hampered by the large amount of salt present and the fact that this was not P-6 excluded extract originally, further efforts indicated the possible presence of RNA in > 75% supernatants. Further work employing P-6 excluded extract* will be needed to confirm this. However, these

* Synthetic boundary ultracentrifugation showed this to be 2.55 S material.

observations were somewhat reminiscent of the pH adjustments mentioned earlier, i.e., salting out like acid addition may liberate RNA -- does it tend to inactivate or cripple ATP synthetase? There is evidence (4) that ammonium sulfate dissociates some coupling factor complexes. Some of the paper chromatographic results obtained relative to the components of >75% salt fractions are shown in Table IV, but much of the data begs interpretation yet. It was also found that more acid-labile P is associated with crude extract than with 0-45% or 45-75% salt fractions. This must also be confirmed with P-6 excluded extract to be sure that the P is not free P_i .

Two experiments were conducted in which extract was prepared specifically to be used for RNA analysis. In both experiments crude extract was freeze-dried immediately and reconstituted in double-distilled water to eliminate any contributions from residual acetone. In one experiment a portion of reconstituted extract was brought to 75% saturation with ammonium sulfate. The resulting precipitate was washed 2x with 75% ammonium sulfate, then redissolved in water for examination of its spectra (Fig. 8). The >75% supernatant was passed through a water-equilibrated Biogel P-2 column from which both included and excluded peaks were collected (qualitatively identical spectra in Fig. 8). Another portion of reconstituted extract was passed directly through the P-2 column; an excluded peak was collected and deproteinized via a Sevag procedure. Typical spectra before and after deproteinization appear in Fig. 9. Spectra were also obtained at various pH's and attempts were made to brominate samples as an aid to identification (5). Bromination led to artifact peaks which were traced to possible origin in the double-distilled water. Some puzzling aspects remain in the work however.

Extract excluded from P-2 was also scanned in the presence of NaCl, trypsin, and dithiothreitol. The hyperchromicity in NaCl is shown in Fig. 8 also. Orcinol test results for the various extract samples in this experiment are presented in Table V.

Finally, using the P-2 excluded extract, cysteine-sulfuric acid tests for DNA and RNA were run; the DNA test was completely negative while the RNA test was strongly positive. In the other RNA analysis experiment all of the extract was freeze-dried and the excluded peak from the P-2 water column was collected and deproteinized. Spectra of bases eluted from paper chromatograms of perchloric acid hydrolyzates are presented in Fig. 10. Spectra of guanine, adenine and uracil are closely identified and associated with the proper R_F values. Material in the cytosine R_F region apparently is not cytosine itself but some relative. In fact, in most cases the spectra correspond better with methylated bases. There is evidence that the hydrolysis was not quite complete since there seemed to be some oligonucleotides on the paper. Of particular interest is a material closely related in spectra to N,N-dimethyladenine. It was noted also that some spots have absorption above 300 nm where sulfur compounds were suspect in earlier work (cf. report no. 3).

Other experiments indicate that complete MOPS removal may be very difficult to obtain.* Much of this idea stems from experiments which have sought the relationships among various determinations of protein, dry weight and RNA in P-6 excluded extracts and 0-45% salt fractions. Generally speaking, freeze-dry weights of samples excluded from P-2 double-distilled water columns often are about 10x or more higher than can be accounted for by the sum of

* Some of this may be because a small amount of MOPS may remain on P-2 water columns and continue to elute slowly after repeated washing.

protein and nucleic acid determinations. The excess weight appears to be MOPS and can be reduced by dialysis. It is a case of isolating a needle from a haystack in which an extremely minor portion of the haystack comes along with the needle and still makes it difficult to find the needle. The use of MOPS blanks often cures the problem but not in some analyses.

There were two experiments which sought evidence for $AD^{32}P$ formation from polyadenylic acid by extract-catalyzed phosphorolysis with $^{32}P_i$. Neither of these experiments (Fig. 11 and 12) produced any $AD^{32}P$ but in one case (Fig. 11) inspection of chromatograms under ultraviolet light revealed spots which suggested that the polyadenylic acid had been subjected to hydrolase (RNase) action. Elution of the spots and examination of eluate spectra confirmed the apparent presence of AMP and larger oligonucleotides; it is remotely possible that the AMP was labeled but more likely that the resolution between AMP and $^{32}P_i$ was not complete. Chromatograms were run longer in the experiment of Fig. 12 to eliminate this resolution problem but here no ultraviolet-absorbing spots were found (may have been too dilute). This experiment has count in the AMP region; however, reference to a chromatogram of $^{32}P_i$ only (Fig. 15) indicates that count may not be in AMP.

Examination of many experiments in which pancreatic RNase was added to inhibit ATP synthetase revealed that stimulation was more commonly observed than inhibition. As presented in the last report, RNase added after the ATP synthetase reaction had begun was ineffective as an inhibitor and often showed inexplicable stimulation. Experiments on P_i binding by pancreatic RNase (Table VI) and the effects of preincubation of extract with RNase versus the contact with RNase only during the incubation (Fig. 13) indicate that when RNase inhibition is observed it is probably due to P_i binding by the RNase.

Since the P_i concentration in ATP synthetase assays is very low ($10^{-5}M$), it only requires a small amount of P_i binding by RNase to be effective. The fact that results of RNase inhibition studies have varied from almost nothing to complete to stimulation may be explained by RNase to extract protein ratios and the impression that the P_i binding is a rather slow process (the time that RNase and $^{32}P_i$ were together in reaction flasks prior to the addition of extract was not controlled and probably varied considerably). While the stimulation of ATP synthetase by preincubation of extract with RNase still suggests RNA involvement, the manner in which this occurs remains to be elucidated. Note in Table VII that ATPase inhibition by preincubation with RNase is greater than can be explained by P_i binding.

The behavior of ATP synthetase assay controls which lack extract has always been a source of concern in this work although the levels of control values are usually insignificant relative to sample values. A case encountered quite often is that of Fig. 14 in which the zero time no-extract control indicates G-6- ^{32}P formation with negative values developing as the incubation proceeds. The question arises as to whether one should subtract the control curve or ignore it, i.e., does the count in the controls have anything to do with G-6-P and is the control curve an integral part of the sample curve. Investigations conducted into this problem indicate that no-extract controls never contain G-6- ^{32}P . If these samples are collected with the G-6-P peak from the gas chromatograph and rechromatographed on paper, only $^{32}P_i$ is found; extract-containing samples on the other hand show G-6- ^{32}P on rechromatography on paper. However, it has been found that high count $^{32}P_i$ alone chromatographed on paper contains radioactive impurities and that the R_F of

these impurities is influenced by co-chromatography with G-6-P, in some cases to attain a nearly identical R_F with G-6-P (Fig. 15). Something similar may also be happening in the gas chromatography of whole samples where one is attempting to resolve high count $^{32}P_i$ from G-6-P. The negative counts in Fig. 14 have been found to be due to the formation of some unstable labeled species with just a slightly shorter retention time than G-6-P so that count builds up in the "before peak" collection and results in overcorrection of count baseline. The results of an experiment in which gas chromatographic collections were rechromatographed on paper is shown in Fig. 16. In the experiment of Fig. 17 triplicate gas chromatographic collections were made and combined for paper chromatography; this procedure raises the level of $^{32}P_i$ impurities in controls to where they appear on the plots. In brief, radioactive species appearing in no-extract controls are not G-6- ^{32}P (a remote consideration would be that significant chemical exchange is possible between G-6-P and $^{32}P_i$ -- it is difficult to find the source of necessary activation energy unless it could somehow derive from the ^{32}P β particle -- at any rate, results such as in Fig. 15 would seem sufficient to explain the situation without drawing upon this possibility). The relative failure of extract-containing samples to show much buildup of count in the "before peak" collection in gas chromatography suggests that the control curves should not be subtracted from sample curves. It seems likely that extract protein may bind the ^{32}P impurities or at least interfere with their silylation so that they do not appear in the gas chromatogram to any great extent, i.e., reflecting a lack of volatility when extract has been present.

Outlook

The present task is to attempt to bring somewhat circumstantial evidence for RNA involvement in ATP synthesis into sharper focus. There is little doubt that RNA is present and closely associated with both ATP synthetase and ATPase. If it could be shown that ATP synthetase free of RNA remains completely active, then RNA involvement could be ruled out. If, as expected, such ATP synthetase were inactive it is unlikely that addition of isolated RNA alone could effect reconstitution but it may be possible to add RNA + ATP (energy source) followed by ATP removal (molecular sieve?) and assay for ATP synthetase ($^{32}\text{P}_i \longrightarrow \text{AT}^{32}\text{P}$). Proof of complete ATP removal would be difficult but perhaps it could be done with isotope dilution and hexokinase.

It must be recognized that it is very difficult to separate nucleoprotein components without damaging the protein (or vice-versa). RNase would be perhaps the mildest, most selective reagent to remove RNA, but in fact, it begins to appear that RNase cannot really attack the RNA in this nucleoprotein.* Therefore, except for other possible enzymes, harsher treatments must be resorted to such as the pH 5.3 precipitation discussed. It was mentioned that salt precipitation may partially dissociate the RNA; this must be explored further, for example, by exposing 0-45% saturation precipitates (ATP synthetase active) to higher salt concentrations followed by re-assay and analysis for RNA content.

* at least not in a manner which releases the fragments from the protein.

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2. Kaufman, S. J. Biol. Chem 216, 153 (1955).
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4. Van de Stadt, R. J., Kraaipoel, R. J. and Van Dam, K. Biochim. Biophys. Acta 267, 25 (1972).
5. Vankstern, T. V. and Baev, A. A. Spectra of Nucleic Acid Compounds. IFI/Plenum, New York (1968).

TABLE I

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Some Characteristics of the ATP-P_i Exchange
of Whole Mitochondria Versus Extracts

Exchange Assay System	Nonextractable ³² P, cpm ^{a,b}	
	Whole Mitochondria	Extract
Complete	4330 (60)	5702 (200)
Complete - CoA	5124 (57)	180
Complete - succinate	2667 (53)	346
Complete + DNP	366 (56)	6668
Complete + atractyloside	2890 (53)	6501
Complete + RNase	3862 (505)	7122
Complete - CoA + lo pantethine + DTT ^c	4610 (53)	194
Complete - CoA + hi pantethine + DTT ^c	3974 (55)	168
Complete + DTT ^c	3077 (49)	7228
Complete + PCMB	338 (54)	3912

a duplicate means

b values in parenthesis are nonenzymatic controls

c dithiothreitol (DTT) is used to reduce pantethine to pantetheine in situ

TABLE II

Salt Fractionation of Succinic Thiokinase

<u>Salt Fraction</u>	<u>cpm/aliquot</u>
0-70%	1,780*
0-50% after redissolving 0-70%	1,200*
50-57% " " "	1,300*
57-70% " " "	7,415*
No extract control	1,865*
=====	=====
Crude extract	125,375
0-45%	3,050
45-75%	12,125
No extract control	3,925

* duplicate means

TABLE III

PROPERTIES OF THE SOLUBLE ATPase

Extract preparation	Assay variable	P _i liberated (μmoles/h per mg protein)
P-6 excluded extract	complete	2.28, 2.42
	w/o Mg ²⁺	2.42, 2.50
	+oligomycin	2.39, 2.52
	+DNP	2.57, 2.69
0-45% salt fraction	complete	3.10, 2.62
	w/o Mg ²⁺	0.83, 0.90
	+oligomycin	3.03, 3.10
	+DNP	2.69, 2.48

TABLE IV
 Paper Chromatography of > 75% Salt Fractions
 and Crude Extracts

<u>Preparation</u>	<u>R_F</u>	<u>Description</u>
75% supernatant	0.7	U.V. absorbant tongue with fluorescent tail. Tail at pH 1 had $\lambda_{max} = 254$ nm. with shoulders at 249, 262, and 280 nm. After evaporation and reconstitution of the tail material with double-distilled H ₂ O the 249 nm. shoulder was a prominent peak (probably at expense of 280 nm. shoulder) particularly at neutral and alkaline pH's.
75% supernatant concentrated with Lyphogel	0.94	U.V. absorbant
	0.70-0.86	U.V. absorbant tongue surrounded by fluorescent region
	0.65-0.70	U.V. absorbant (may be part of tongue)
	0.5	very positive test for reducing sugar (apparently hydrolyzed sucrose)
Freeze-dried crude extract	0	fluorescent-broad peak at 255 nm; 280 nm. shoulder
Crude extract aged then freeze-dried	0.1-0.3	peak at 282 nm.
Freeze-dried crude extract 1NHCl, 100°C, 10 min.	0.5-0.6	peak at 282 nm; 284 nm. at higher pH's
	0.2-0.3	peak at 254 nm. with shoulder at 249 nm.
Freeze-dried crude extract 1NHCl, rm. temperature, 16 hrs.	0.5-0.6	yellow spot; peak at 283 nm., minor peaks at 225 and 346 nm.

Note: Both sucrose and MOPS may contribute artifacts in this data and not all observations recorded here.

TABLE V

Orcinol Test Results

<u>Sample</u>	<u>Total μg orcinol-positive material per 10 ml of original crude extract</u>
A - Freeze-dried crude extract excluded from P-2	4267
B - deproteinized A	3600
C - Freeze-dried crude extract, reconstituted and ppt'd by 75% salt saturation	930
D - supernatant from C excluded from P-2	1292
E - supernatant from C included on P-2	3960*

* sucrose contribution probable since orcinol peak here at 615 nm. rather than usual 666 nm.

TABLE VI

 P_i Binding by RNase

<u>Sample</u>	<u>P_i remaining unbound after 1 hr. μg^*</u>
P_i only	92.0 (theoretical = 93.0)
P_i + extract (210 μg protein)	94.0
P_i + RNase (1 mg)	88.0

* duplicate means

TABLE VII

Stimulation and Inhibition of ATPase

<u>Sample</u>	<u>P_i released from ATP $\mu g/hr.$ *</u>
ATPase	138.0
ATPase + RNase (preincubation)	70.5
ATPase + DTT	168.0
ATPase + PCMB	123.0

* duplicate means corrected for incubated
no extract control

Figure 1

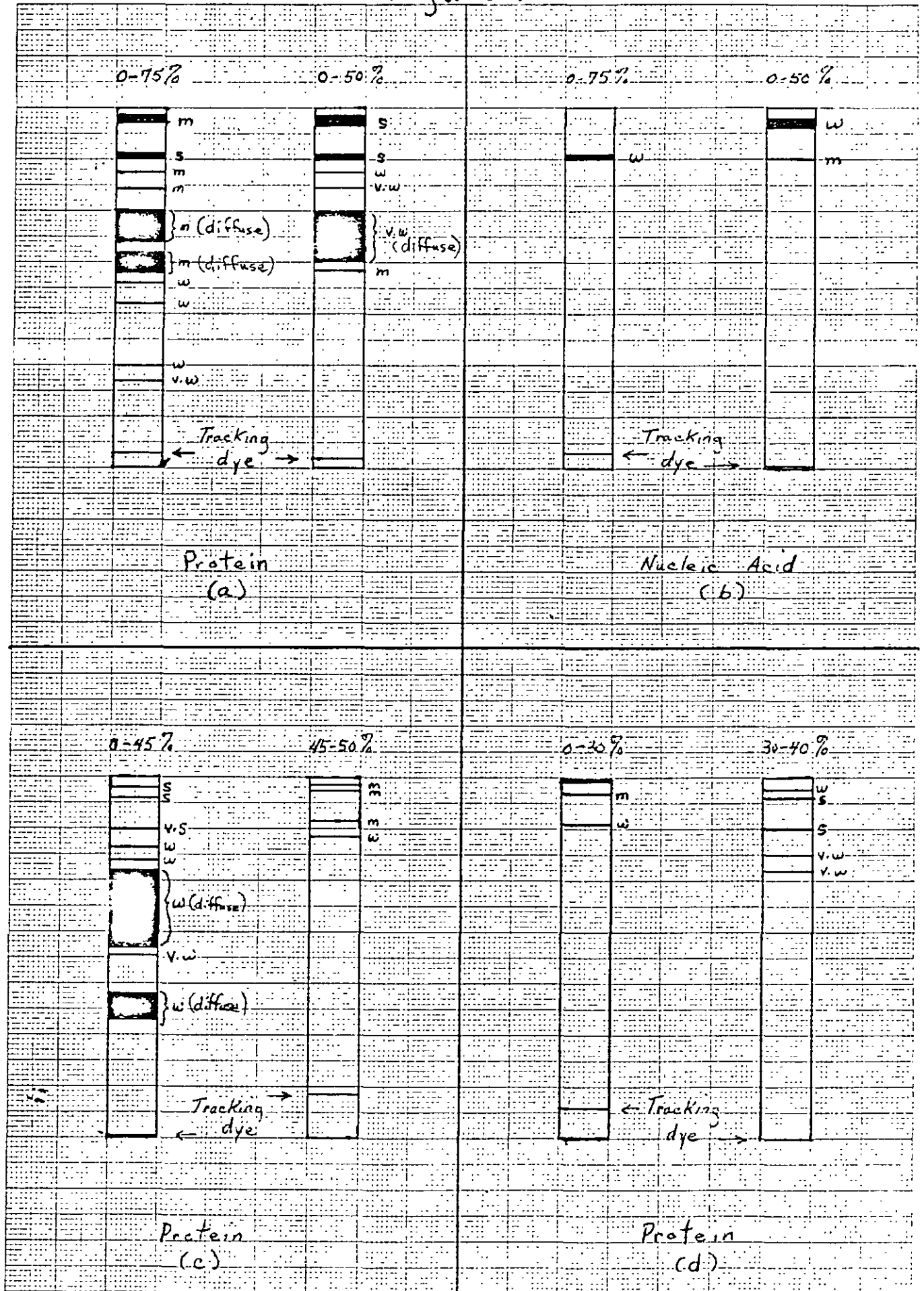
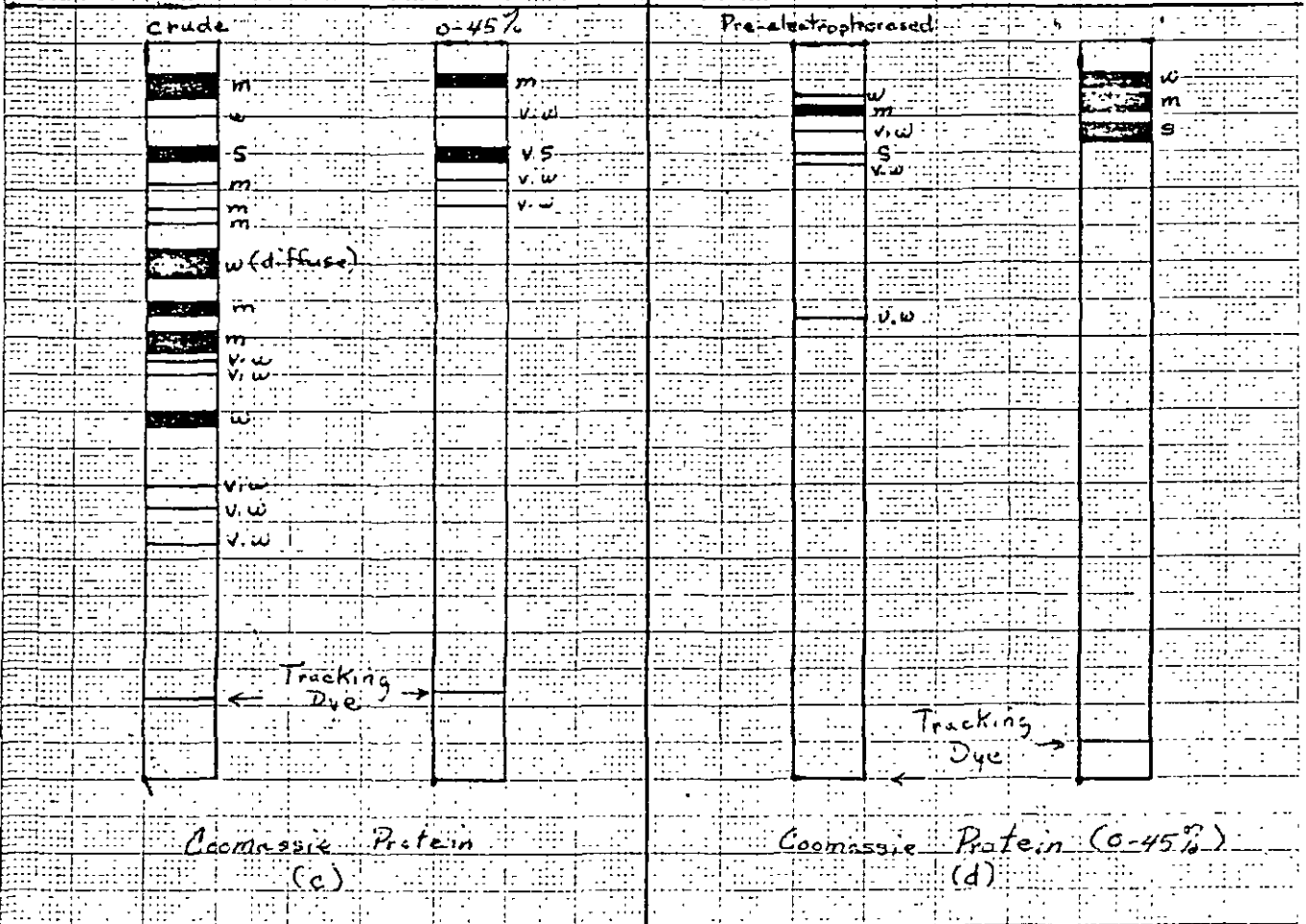
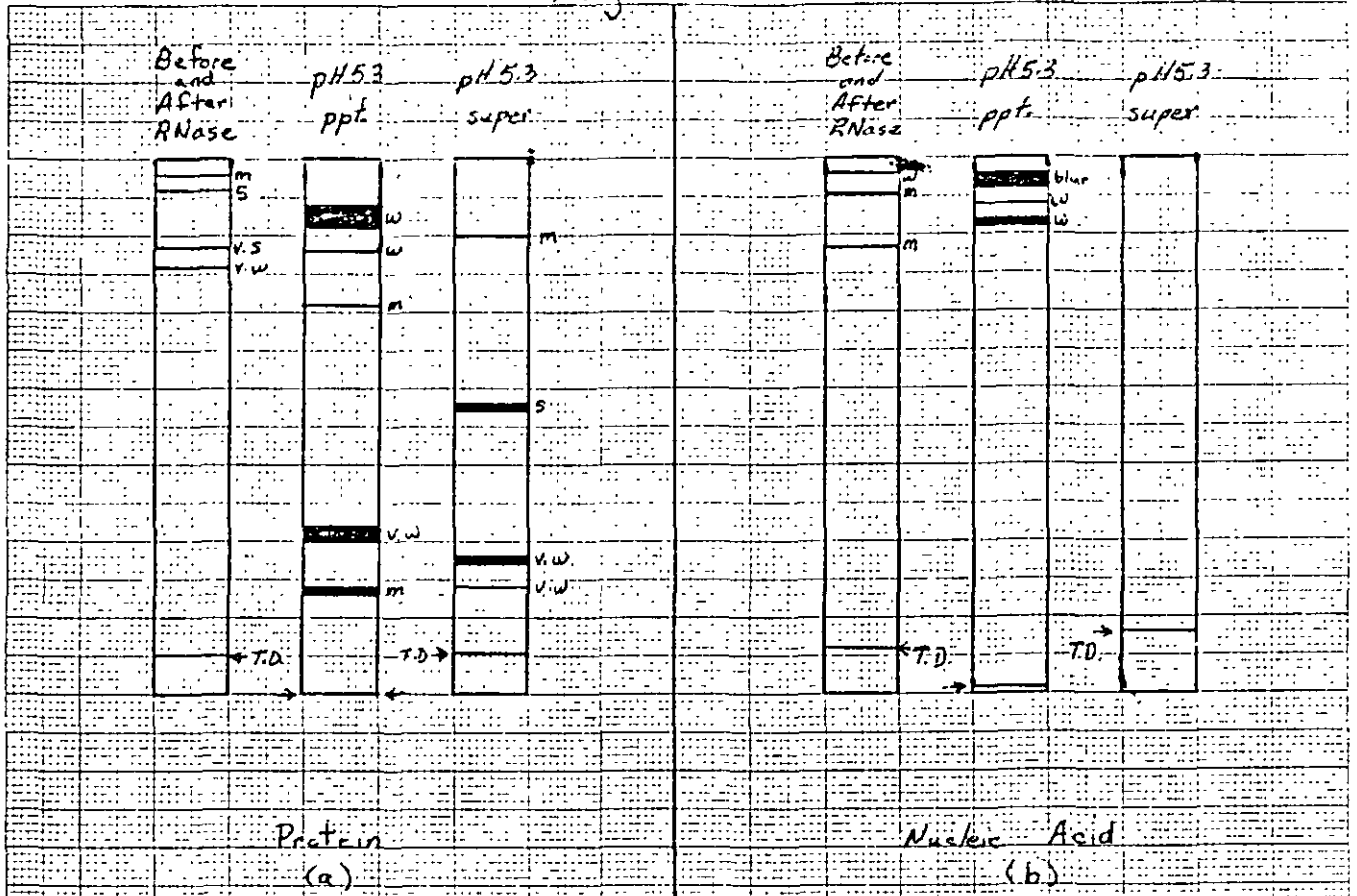


Figure 2



G-6-³²P FORMATION FROM ³²Pi

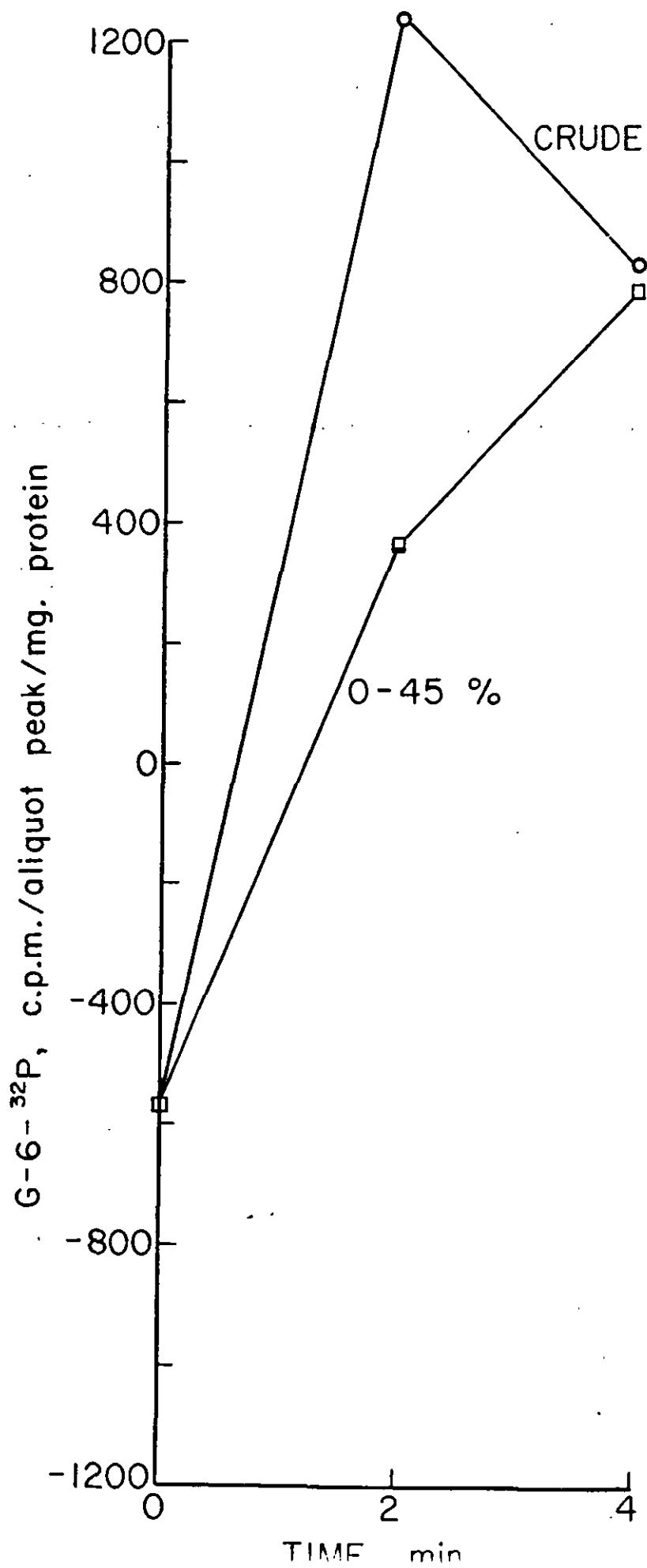
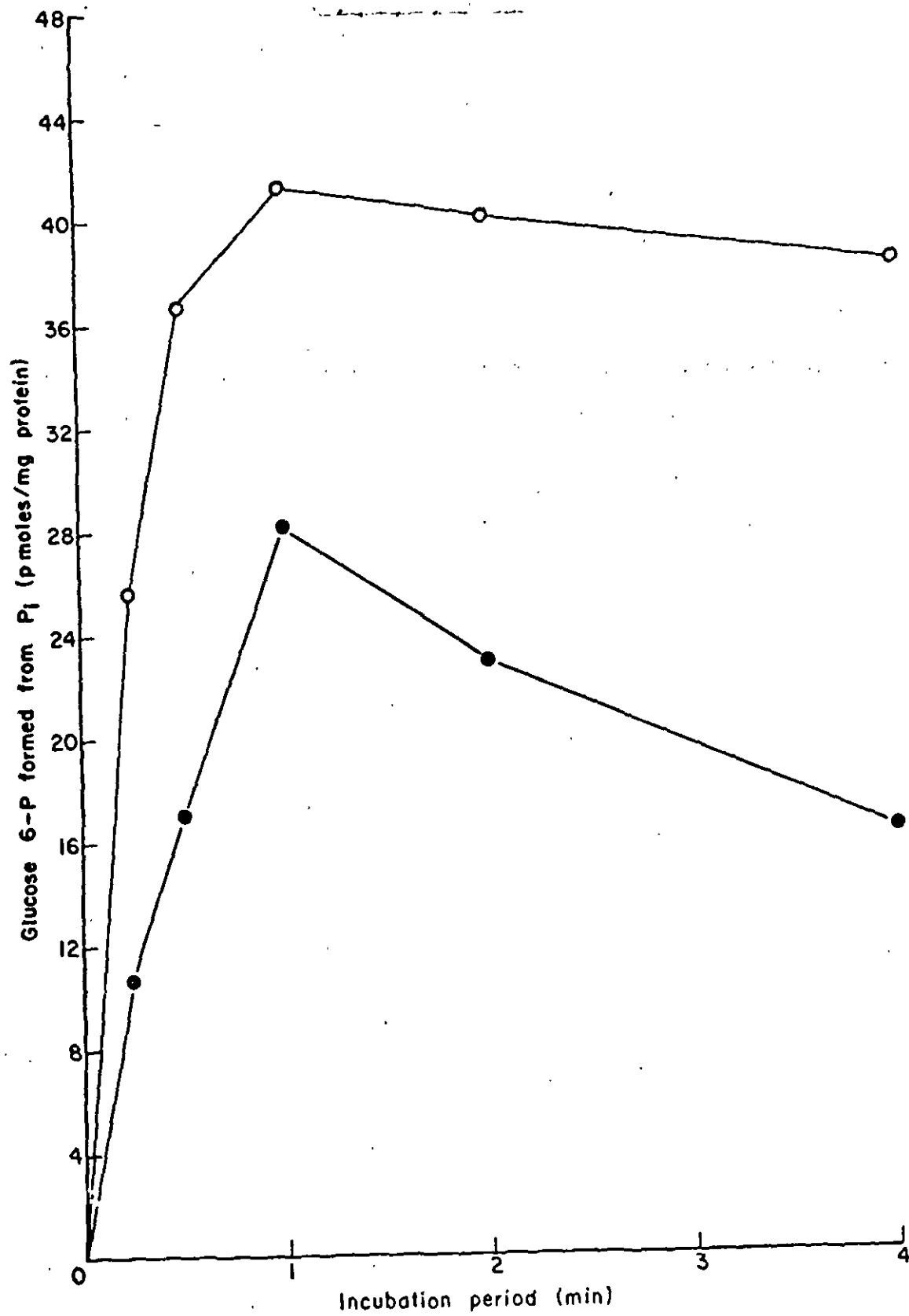


Figure 3

Figure 4



G-6-PASE AND ISOMERIZATION

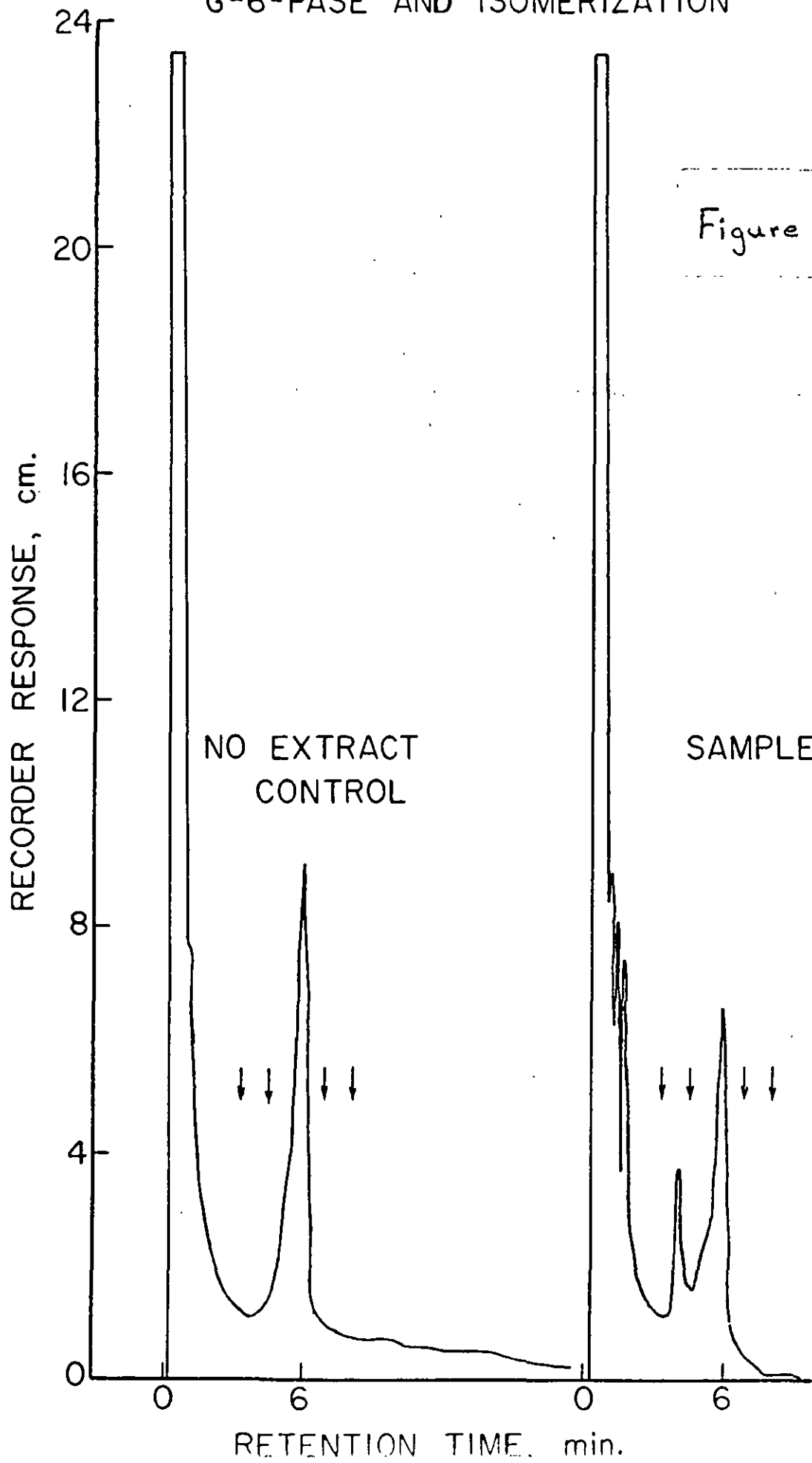


Figure 6

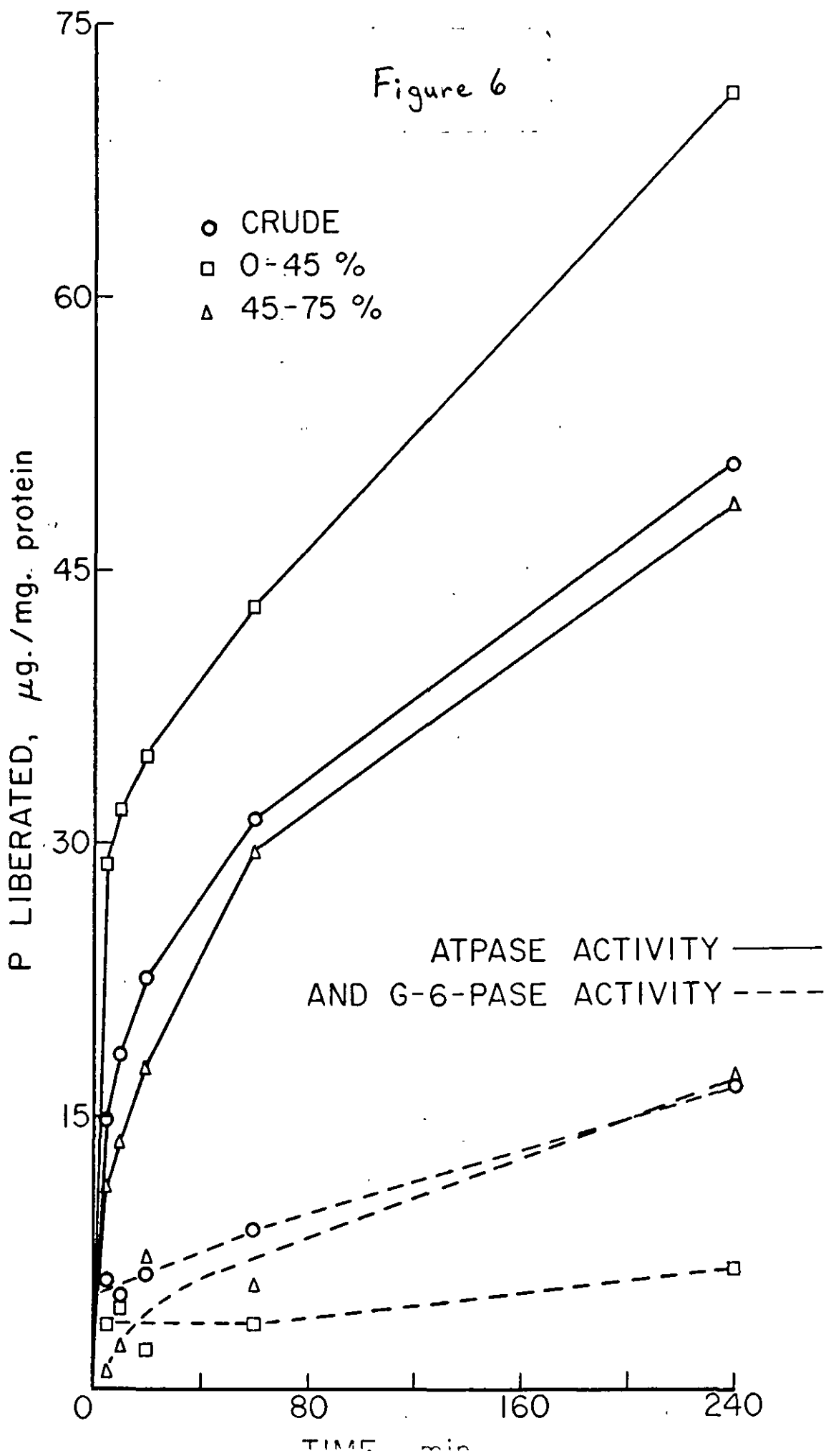
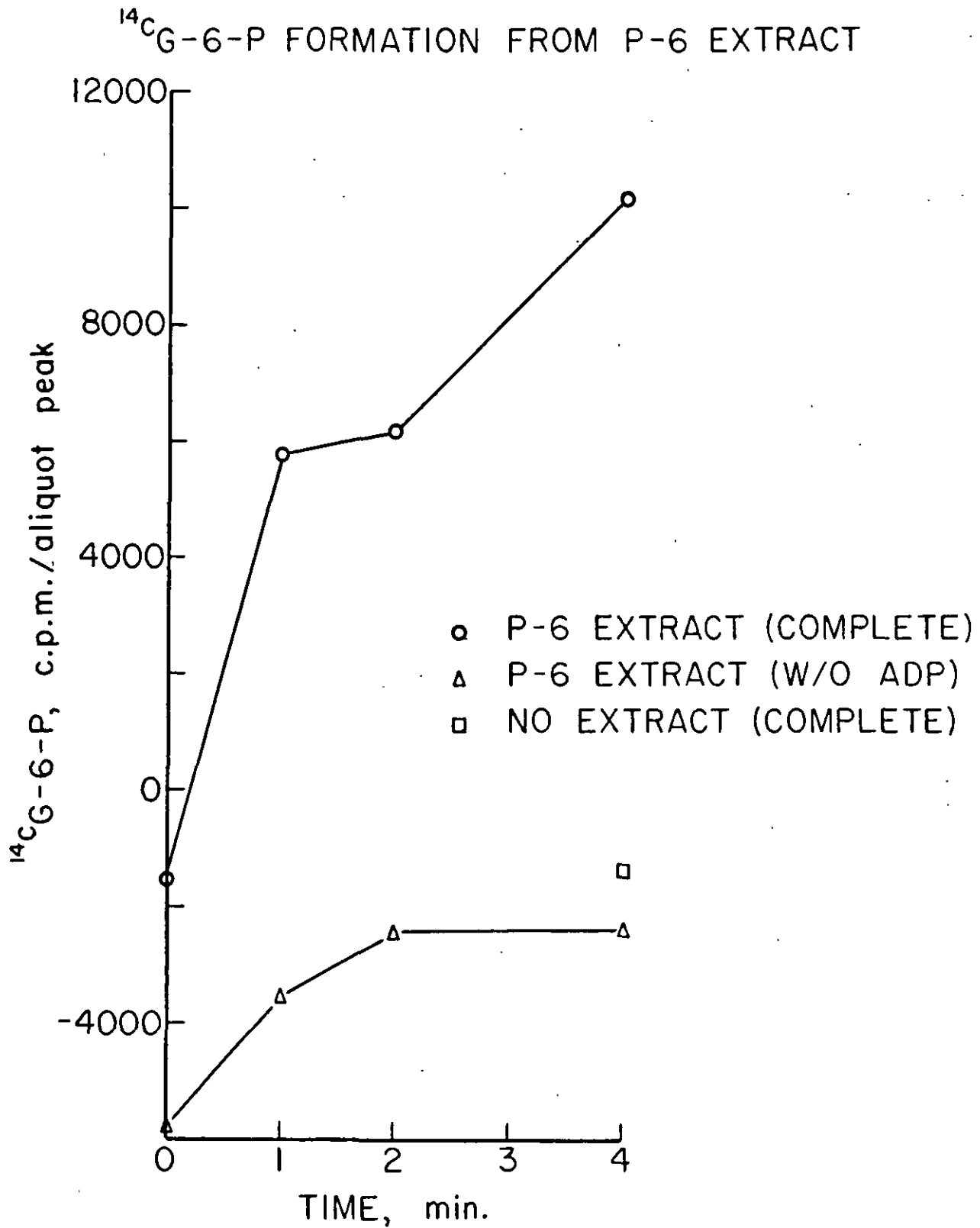


Figure 7



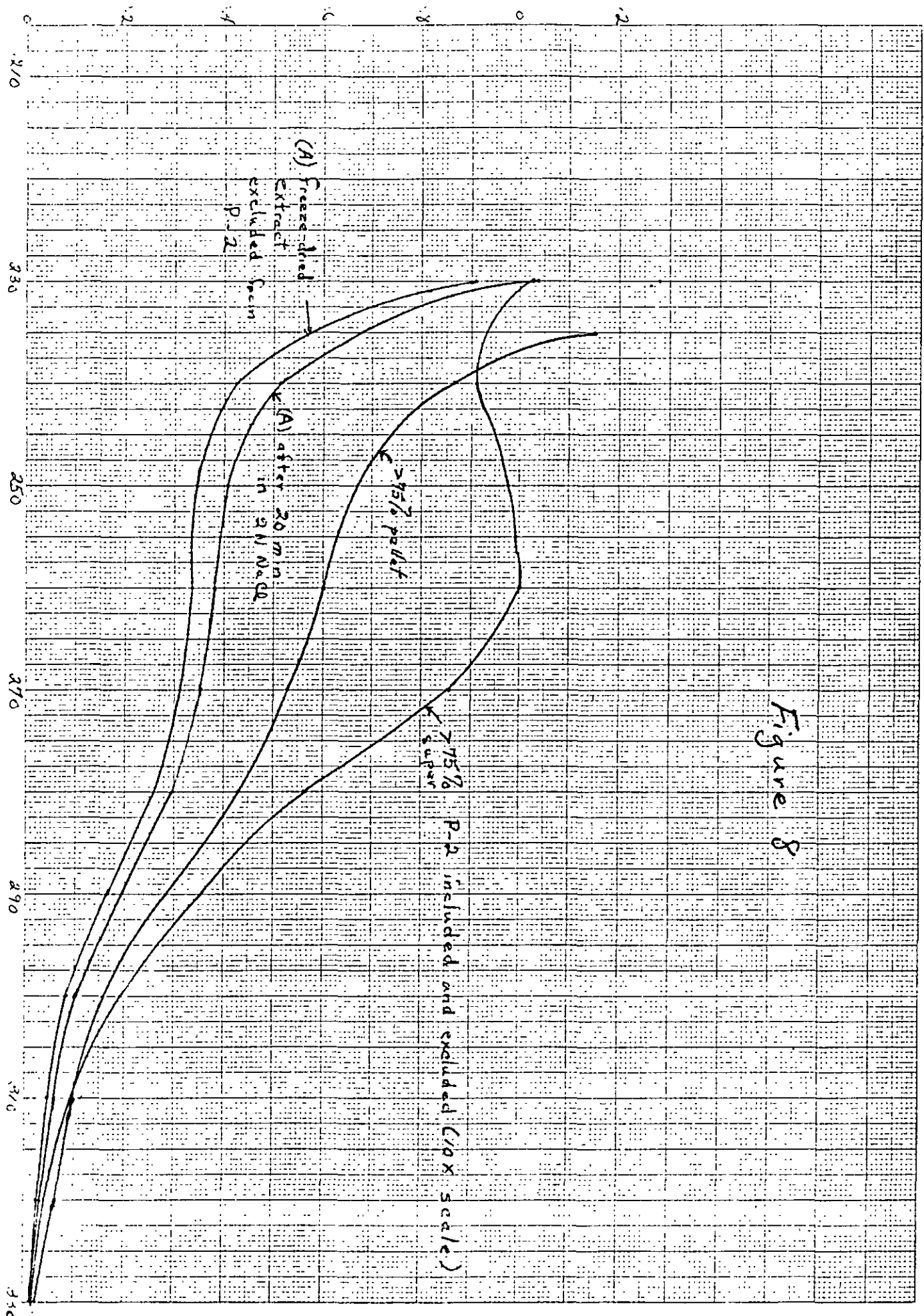
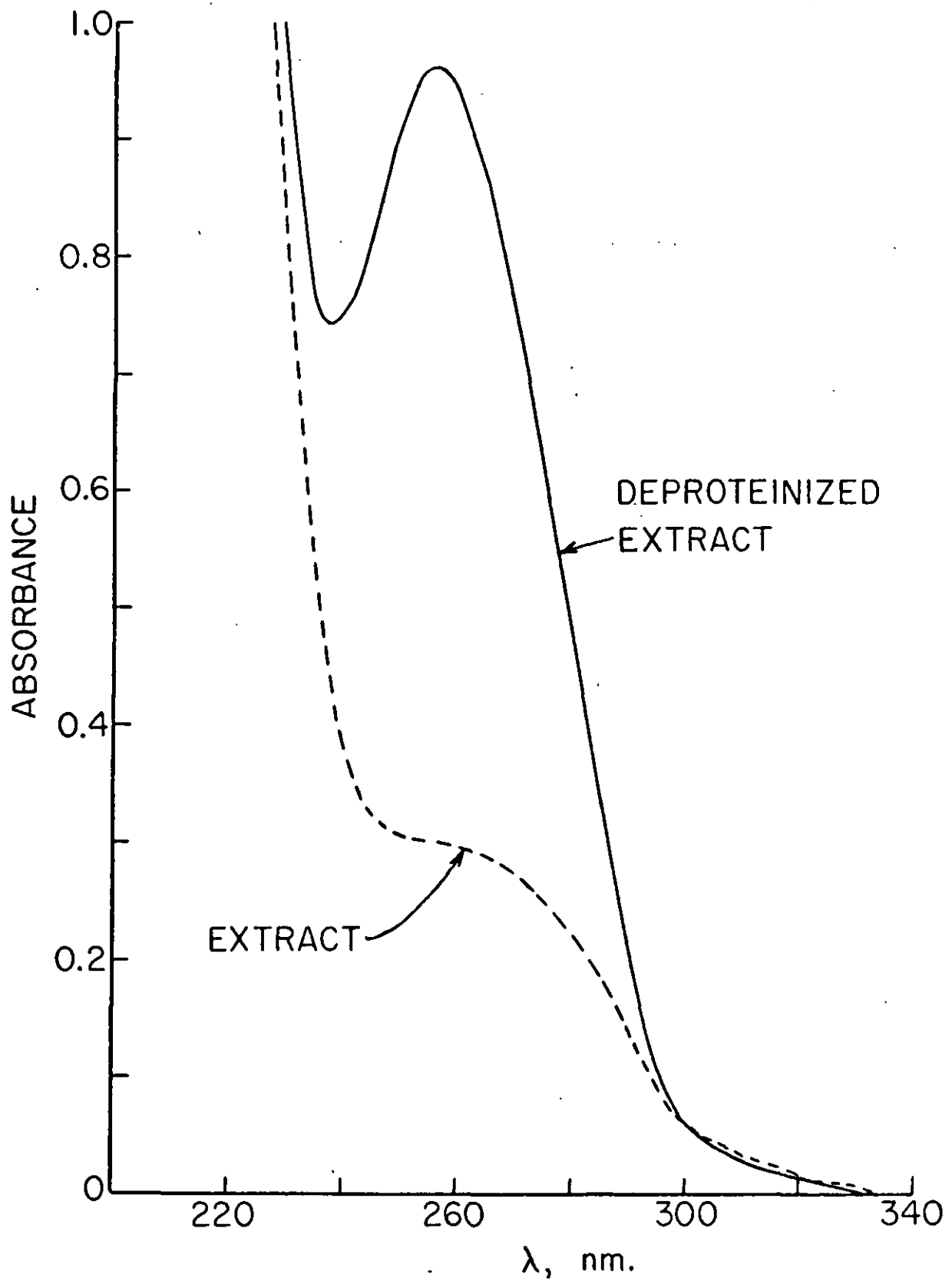


Figure 8

Figure 9

U.V. SPECTRA IN WATER



Absorbance

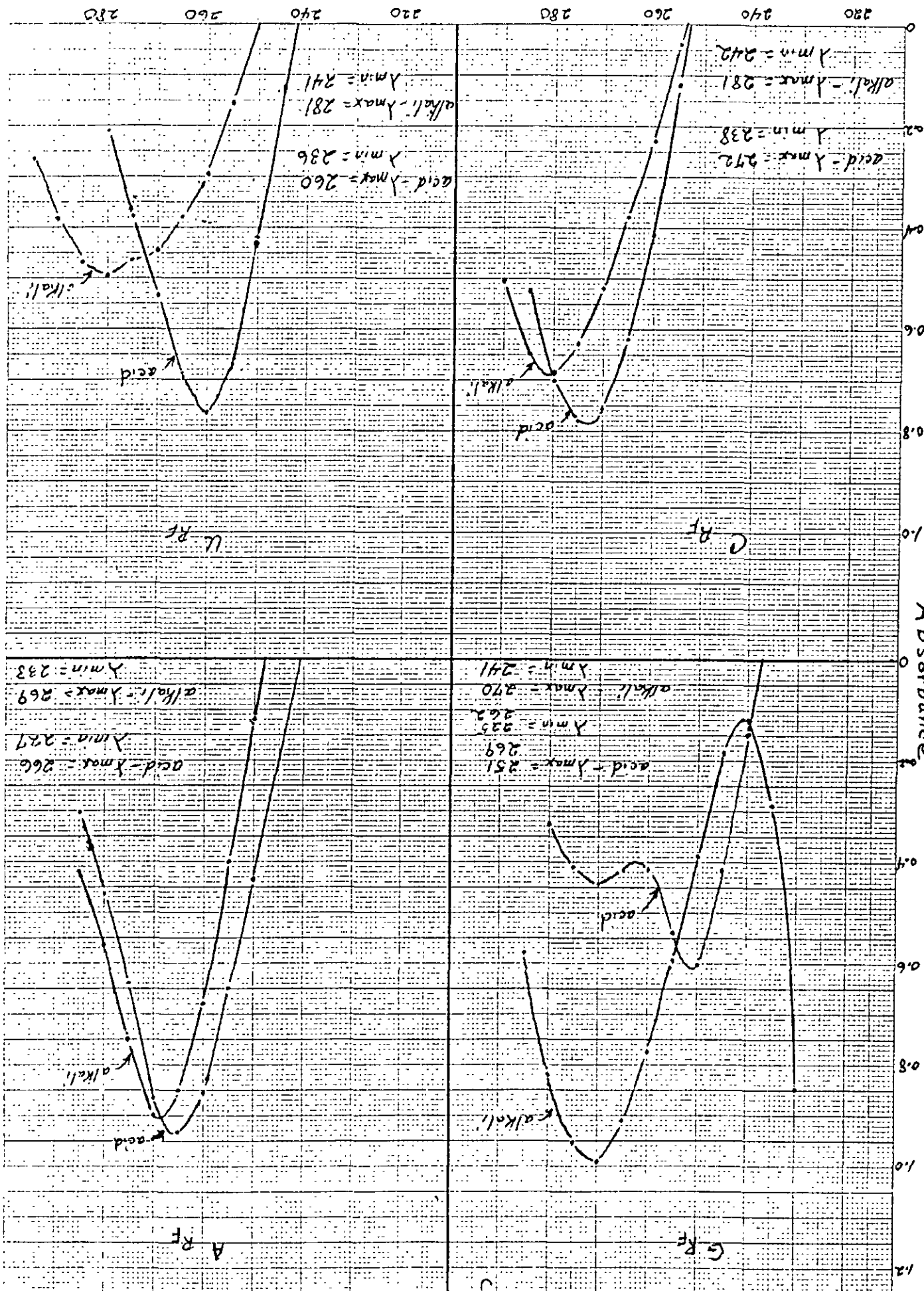


Figure 10

K₀E 10 X 10 TO THE CENTIMETER 46 1510
10 X 25 CM. MADE IN U. S. A.
KEUFFEL & ESSER CO.

32p, cpm x 10⁻³

Figure 11

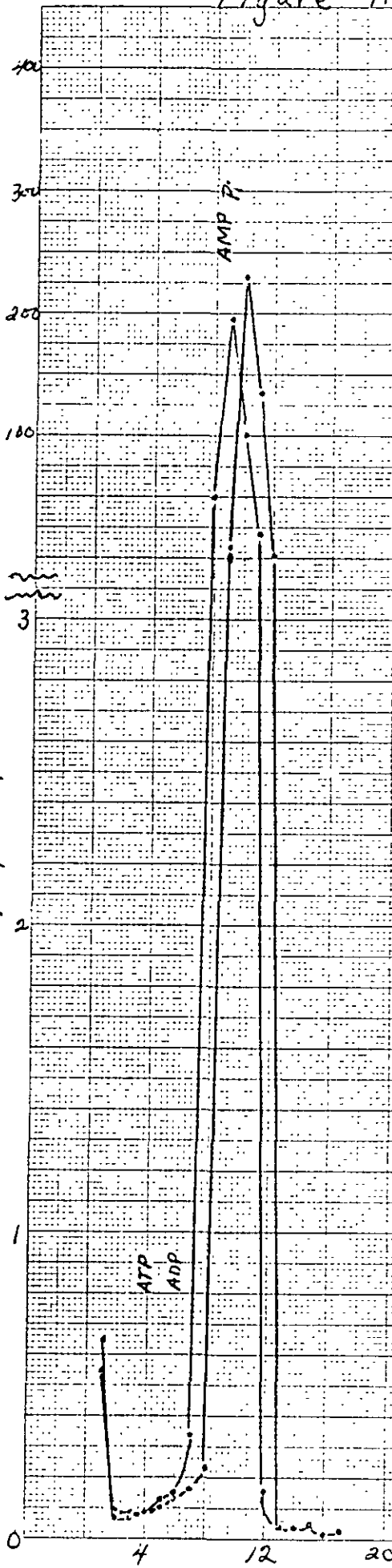


Figure 12

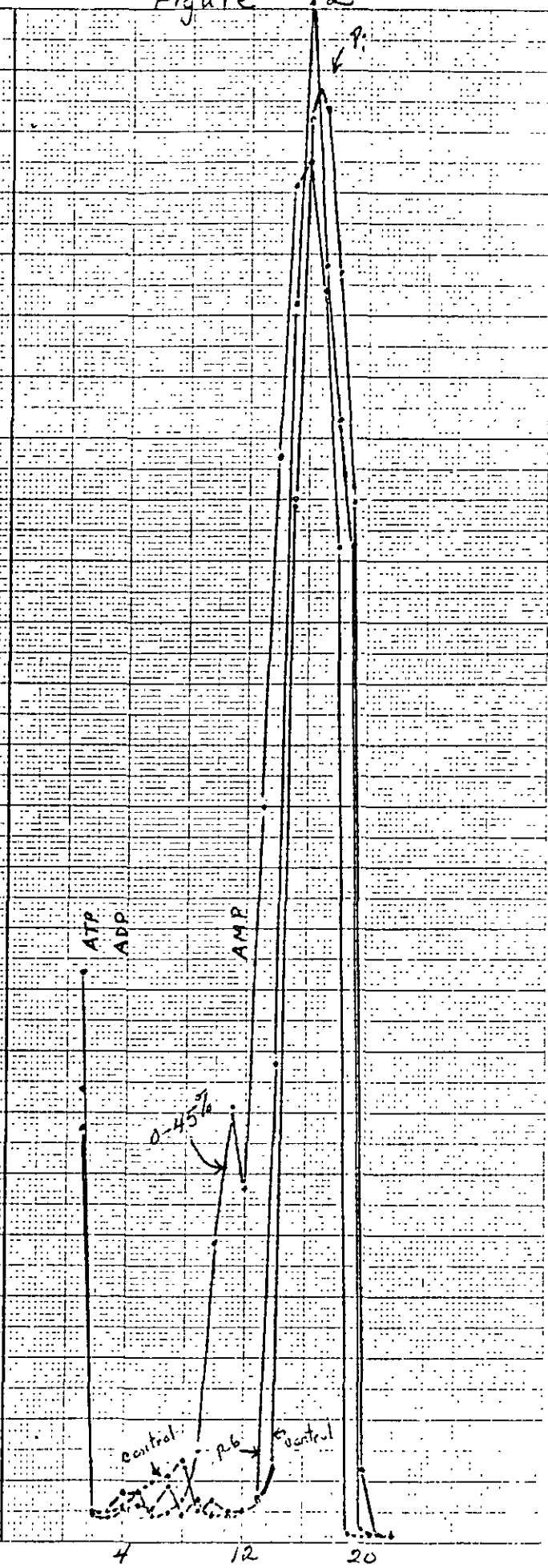


Figure 13

K&E 10 X 10 TO THE CENTIMETER 46 1510
MADE IN U.S.A.
KEUFFEL & ESSER CO.

G 6-³²P Formed (cpm/aliquot peak/mg $\times 10^{-3}$)

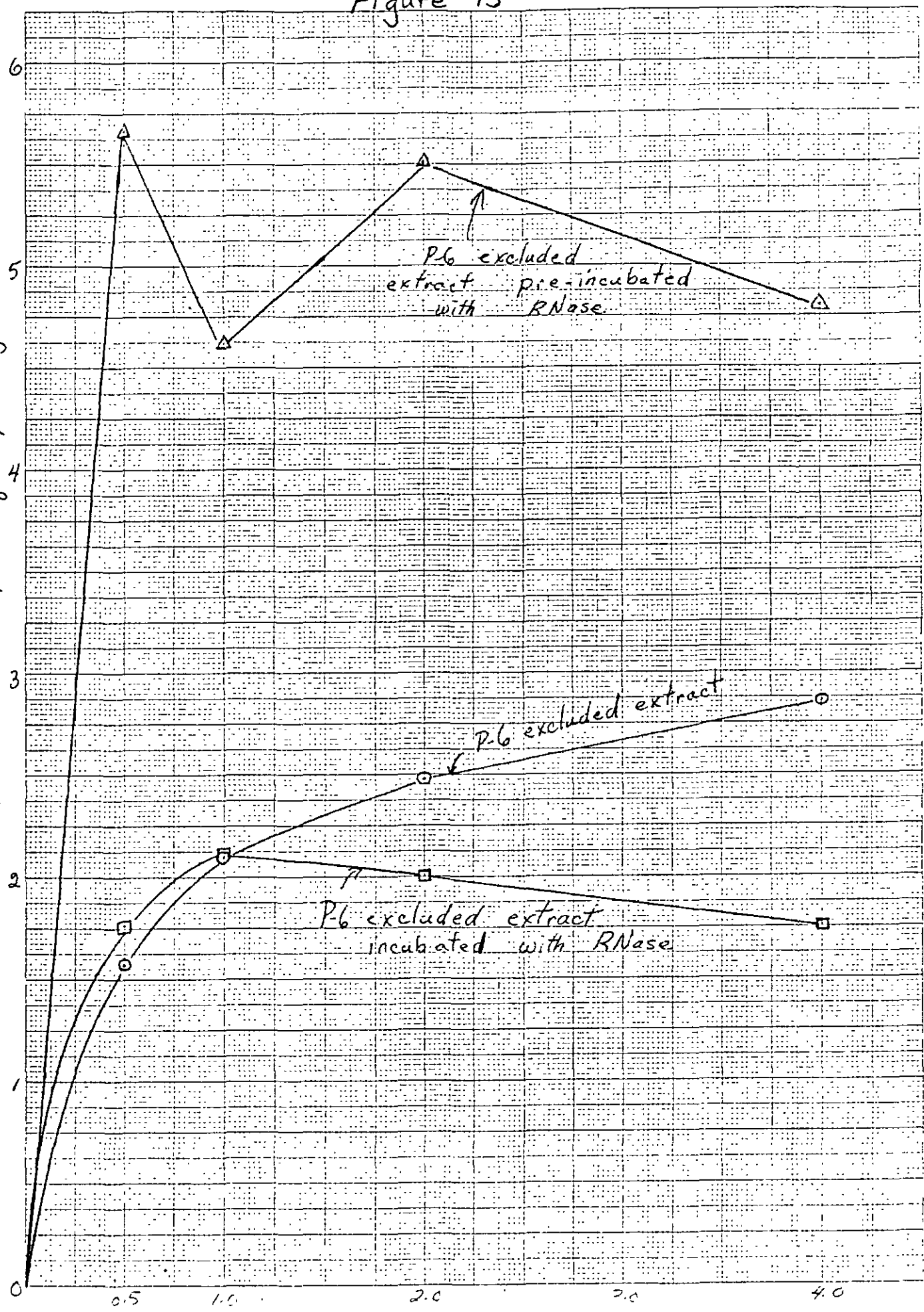


Figure 14

G-6-³²P FORMATION FROM ³²Pi

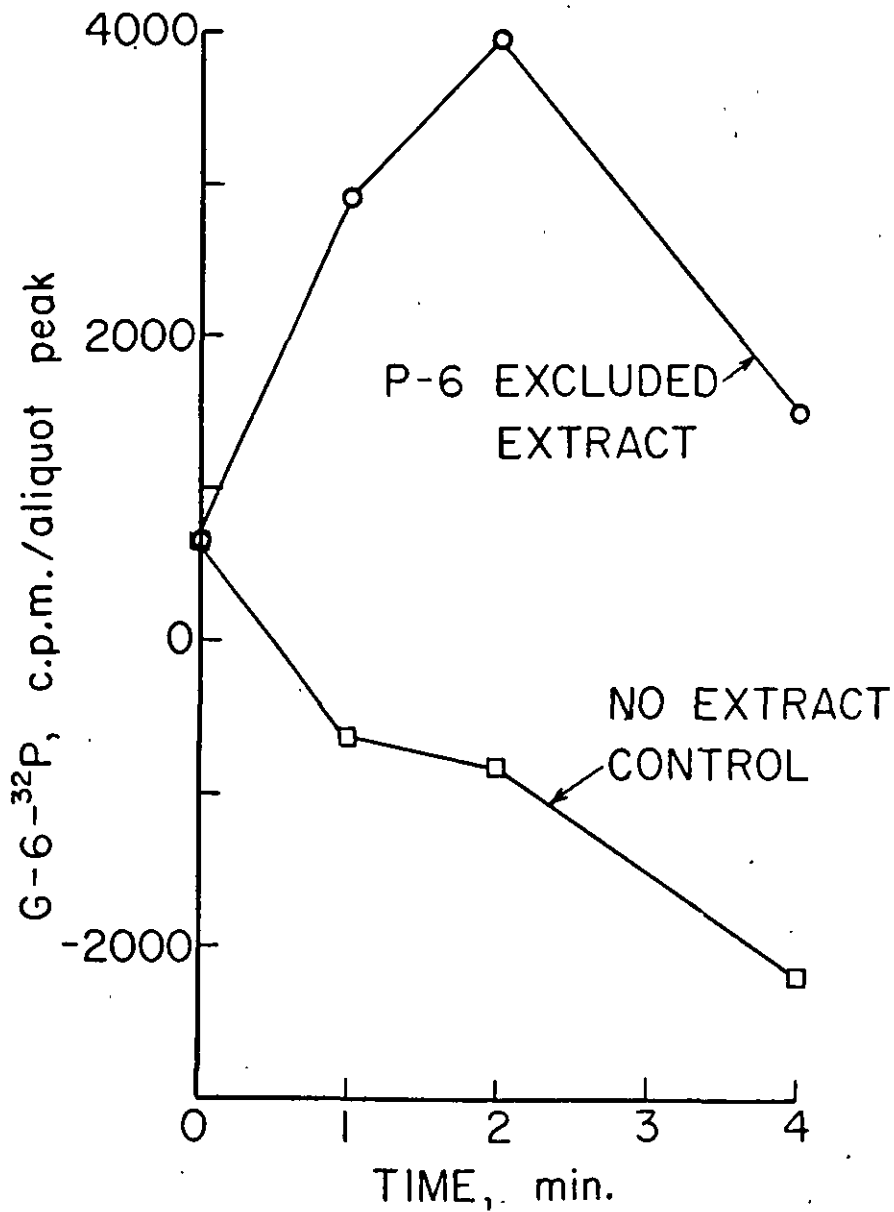


Figure 15

K⁰E 10 X 10 TO THE CENTIMETER 46 1510
MADE IN U.S.A.
KEUFFEL & ESSER CO.

^{32}P , cpm $\times 10^{-2}$

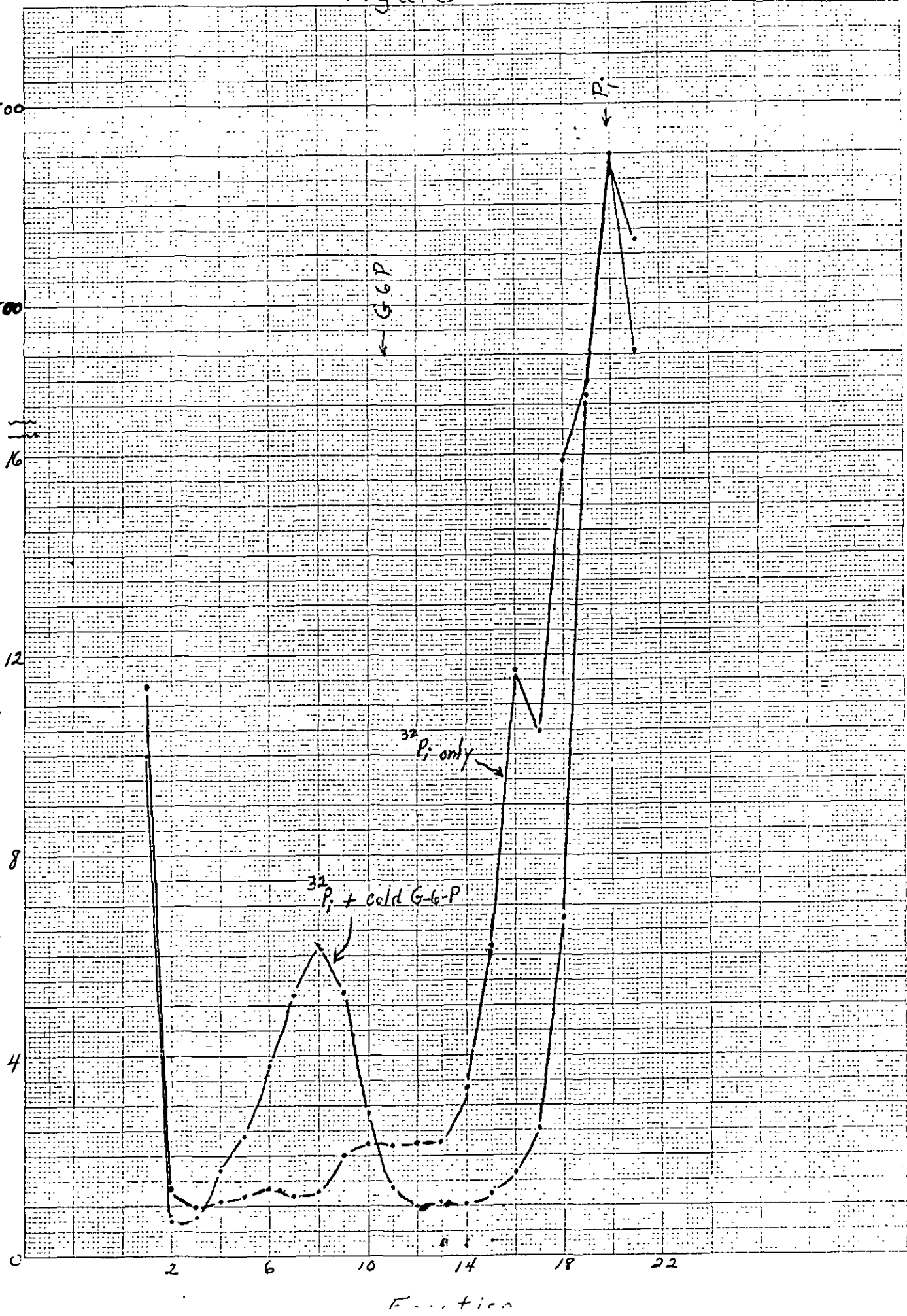
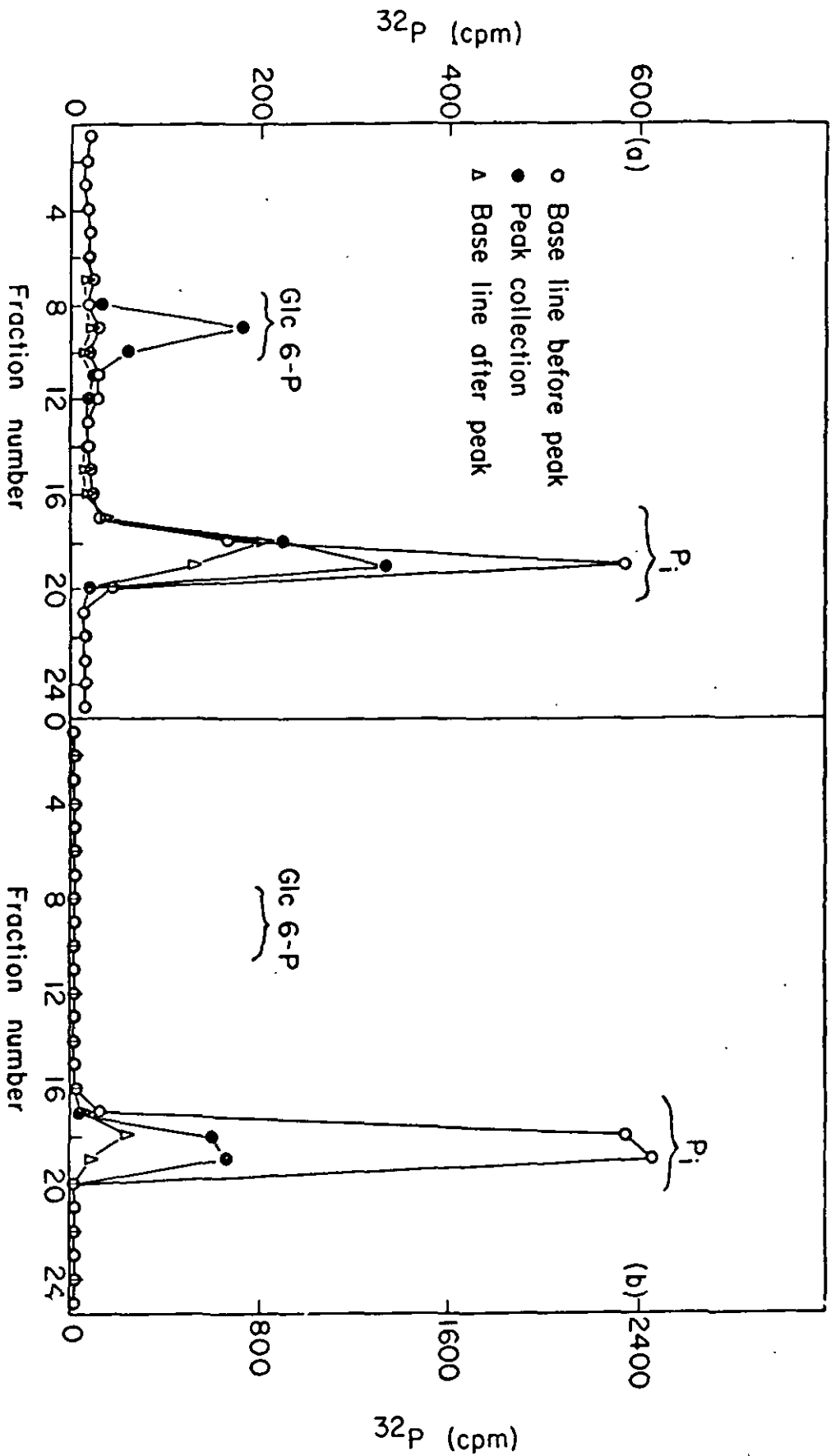


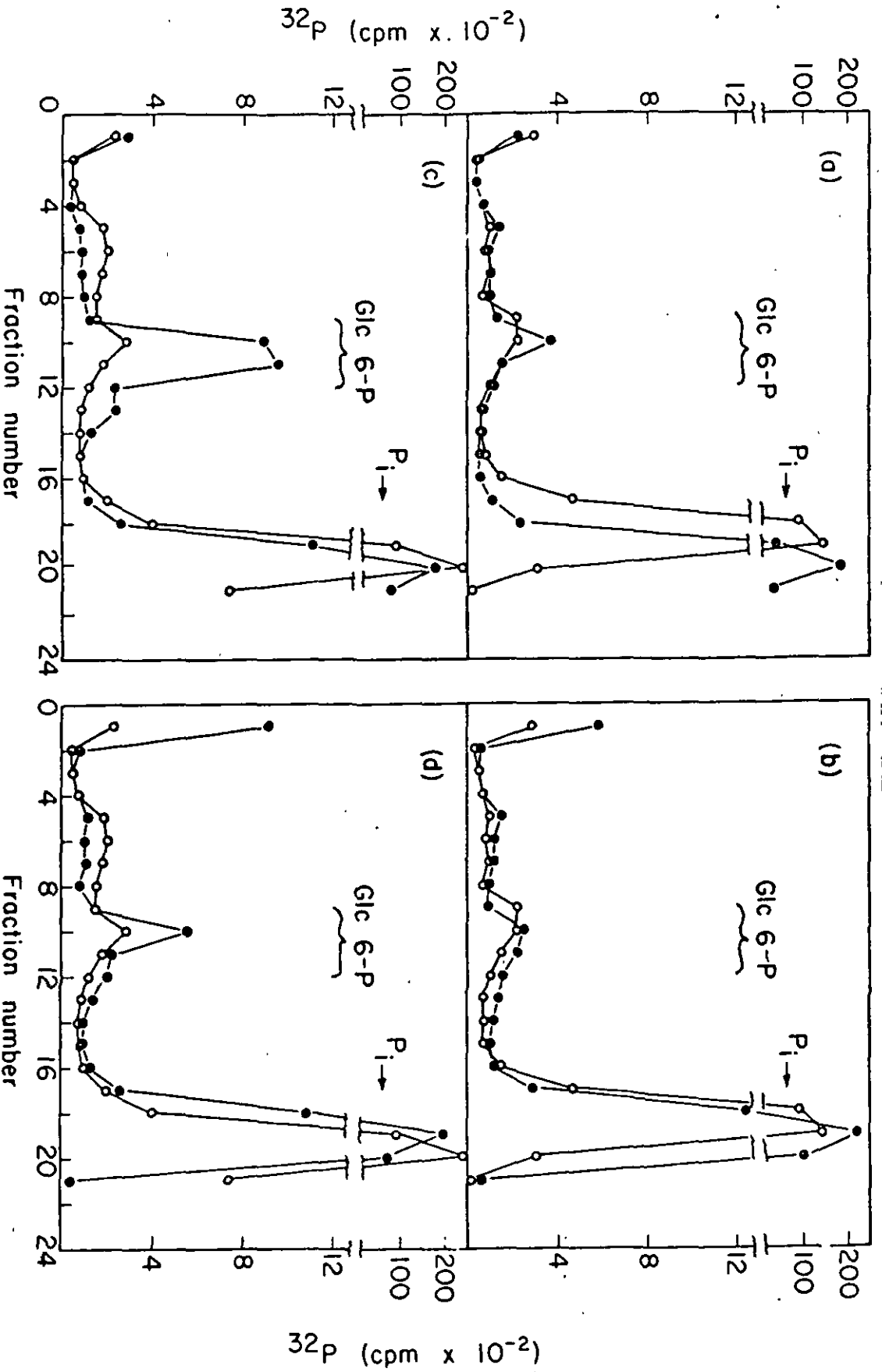
Figure 16



(a) = sample

(b) = control

Figure 17



(a) zero time
(c) one min.
P-6 extract

(b) zero time
(d) one min.
0-45% cut

● samples
○ no-extract controls

^{32}P (cpm $\times 10^{-2}$)

^{32}P (cpm $\times 10^{-2}$)