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Activation In Vitro of Transfer RNA-Guanine Ribosyltransferase by Protein Kinase C

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ACTIVATION IN VITRO OF TRANSFER RNA–GUANINE RIBOSYLTRANSFERASE BY PROTEIN KINASE C

by

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ABSTRACT

ACTIVATION IN VITRO OF TRANSFER RNA–GUANINE RIBOSYLTRASFERASE BY PROTEIN KINASE C

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Director: Dr. Mark S. Elliott

The purpose of this study was to isolate and purify the enzymes, transfer RNA–guanine ribosyltransferase and protein kinase C, and to determine whether the phosphorylating enzyme activates the insertion enzyme *in vitro*. Transfer RNA–guanine ribosyltransferase lost activity within several days after isolation and total, complete reactivation was accomplished in the presence of protein kinase C. This demonstrated that ribosyltransferase's instability is related to the degree of its phosphorylation. It is proposed that modification of tRNA is controlled by protein kinase C. Deactivation of the insertion enzyme leads to hypomodified tRNA which in turn is associated with neoplasia. Potential use of these results could be helpful in unraveling the mechanism of induction of cancer.

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INTRODUCTION

Transfer RNA–Guanine Ribosyltransferase

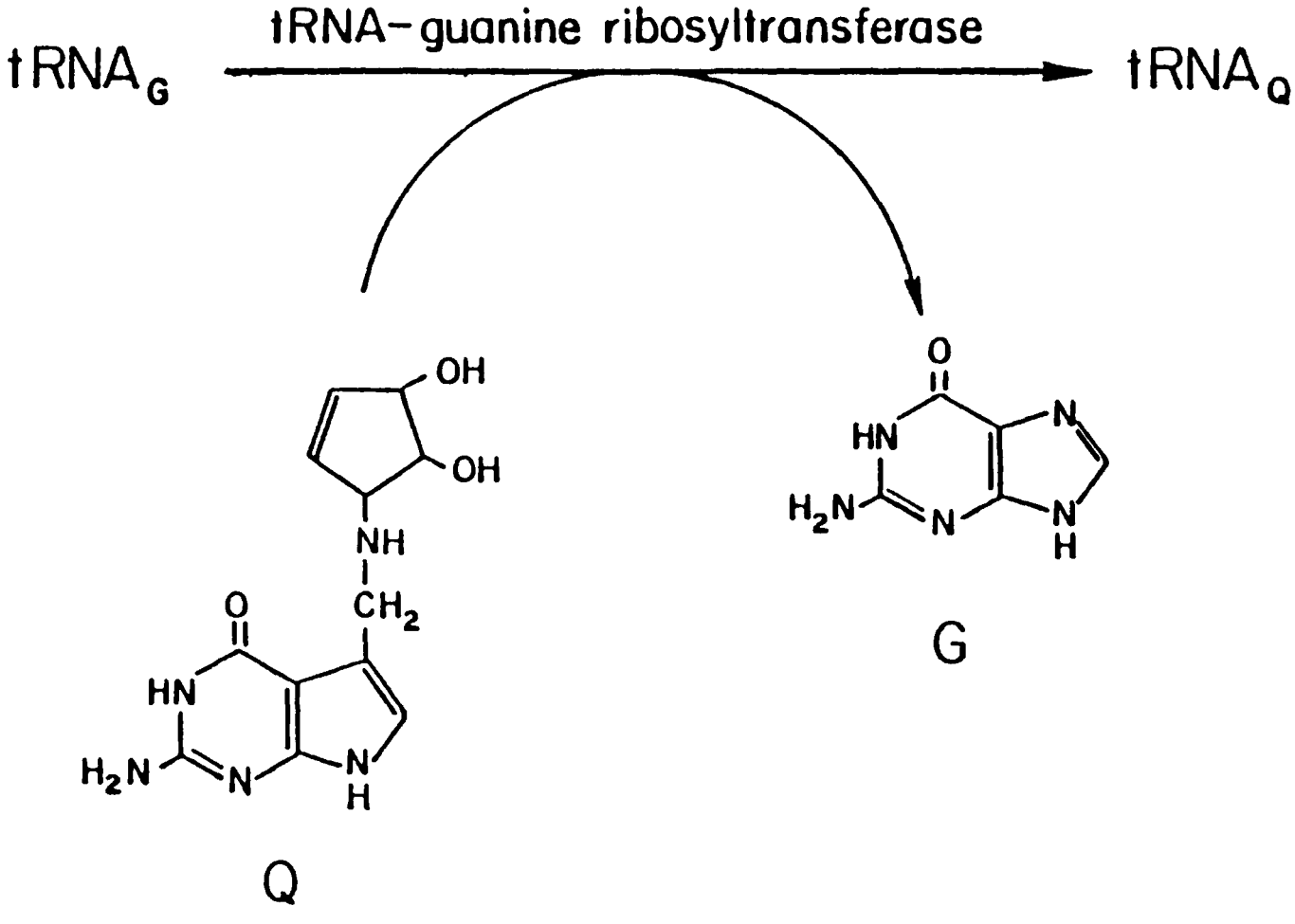
Queuosine is one of the most complex and unique modified guanosine analogs found in tRNA. Since its discovery in 1967, substantial interest has been demonstrated concerning its distribution, biosynthesis and function. It was found in the first position of the anticodon of *E. Coli* $tRNA^{Tyr}$ by three independent groups during the nucleotide sequence analysis of this tRNA [Rajbhandary *et al.*, Goodman *et al.* and Doctor *et al.*(1)]. Queuosine was found later to be present in *E. Coli* $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Asp}$, in the first position of the anticodon (2). It was concluded that all *E. Coli* tRNAs that recognize U and C in the third position of the codon and A in the second position of the codon contain queuosine. Queuosine is the only nucleoside of ribonucleic acid where the purine skeleton has a 7–deaza structure. In eukaryotic cells, a galactose–containing queuosine of $tRNA^{Tyr}$ was discovered and a mannose–containing queuosine was found in $tRNA^{Asp}$ (3, 4). In 1979 the three dimensional structure of queuosine was determined by X–ray crystallography and its conformation was analyzed by NMR studies (5). Queuosine is found in many different organisms that include rat, rabbit, fish, wheat germ, vertebrates, brachiopods, plants and bacteria. Yeast, however, is free of queuosine; it cannot synthesize it or incorporate exogenous queuine into its tRNA (6).

The formation of queuosine in tRNA is accomplished by a post–transcriptional modification mechanism in which the enzyme tRNA–guanine

ribosyltransferase (QRTase) exchanges a guanine base for queuine in an irreversible reaction in the first position of the anticodon of tRNA accomodating the amino acids tyrosine, histidine, asparagine and aspartate (7). The insertion enzyme catalyzes this reaction by cleavage of the N–C glycosidic bond between the base and the ribose without breakage of the phosphodiester bond and without any energy source such as ATP (Figure 1). The properties of tRNA–guanine ribosyltransferase vary with the sources of the enzymes. The enzyme has been purified to homogeneity (8, 9, 10) from rabbit erythrocytes, wheat germ and *E. Coli.* The enzyme from rabbit erythrocytes has a molecular weight of 104,000 Kd, and consists of two subunits of molecular weight of 60,000 Kd and 43,000 Kd (11). The wheat germ enzyme has a molecular weight of 140,000 Kd, consists of two 68,000 Kd subunits, and requires magnesium ions for activity. The *E. Coli* enzyme has a molecular weight of 43,000 Kd, is a single polypeptide, and is stimulated by magnesium ions. The molecular weight of the enzyme from rat liver is 80,000 Kd and its K_m for guanine is 8.3×10^{-7} M, and for queuine is 3×10^{-7} M. Even though the insertion enzyme from rat liver has a greater affinity for queuine than for guanine, the insertion of guanine is a reversible reaction where that of queuine is an irreversible one. The pH optima for all of the enzymes are similar (11). The mammalian and plant enzymes insert queuine where the bacterial enzyme uses the biosynthetic precursor 7–(aminomethyl)–7–deazaguanine rather than queuine as substrate (1).

Farkas *et al.* showed that mice cannot synthesize queuine and must get it exogenously, like vitamins, and that queuine from common plant and animal food products is effectively utilized for the formation of queuine containing tRNA *in vivo* (11). Keeping in mind that intestinal bacteria are able to synthesize queuine, experiments were carried out with germfree mice. These experiments indicated that

Figure 1. The irreversible exchange reaction of the base guanine for the base queuine in the first position of the anticodon of tRNA accomodating the amino acids tyrosine, histidine, asparagine and aspartate.


tRNA-guanine ribosyltransferase
tRNA$_G$
tRNA$_Q$
OH
OH
NH
CH$_2$
O
HN
H$_2$N
N
N
H
Q
O
HN
H$_2$N
N
N
N
H
G

there was a depletion of queosine–modified tRNA from the livers of those mice, including all four isoacceptors; $tRNA^{Asn}$, $tRNA^{His}$, $tRNA^{Asp}$ and $tRNA^{Tyr}$. Their data supported the idea that the intestinal flora were capable of providing queuine and restoration of queuine could be accomplished by adding queuine as a dietary supplement or by injecting it into the animal intraperitoneally (11).

Transfer RNA's most important role is to translate the genetic code. Queuine modification would be considered a structural change that could result in alterations of codon–anticodon recognition and, therefore, a change in protein synthesis. Known inhibitors of tRNA–guanine ribosyltransferase are 7–methyl guanine, and the purine antimetabolites 6–thioguanine and 8–azaguanine. Each are excellent substrates for the insertion enzyme *in vitro* and *in vivo* (1). It has been reported by Elliott and Trewyn that 7–methyl guanine induces queuine hypomodification of tRNA in chinese hamster embryo cells and enhances their chemical transformation *in vitro*. Two inhibitors were tested, 7–methylguanine and 1–methylguanine, to test queuine base modification of normal chinese hamster cells. It was concluded that a concentration of 10 μM of 7–methylguanine resulted in profoundly Q–deficient tRNA. This was not true for 10 μM of 1–methylguanine. It was suggested that, since malignant tissues contain methyltransferases and cancer patients excrete elevated methylated derivatives of tRNA catabolites, queuine hypomodification of tRNA is closely related to neoplasia (12). Muralidhar *et al.* tested different purine analogs as inhibitors that result in queuine hypomodification of tRNA. Development of a small–scale assay was used to test cultures of chinese hamster embryo cells of their incorporation of [^{3}H]–dihydroqueuine in tRNA in the presence and absence of different inhibitors. In their results 7–methylguanine, 6–thioguanine, 8–azaguanine, and xanthine were potent inhibitors that resulted in no detectable incorporation of radiolabeled dihydroqueuine. Allopurinol,

oxypurinol, 6–ethylmercaptopurine had a minimal effect in blocking the incorporation of [^{3}H] dihydroqueuine. It is noteworthy that all purine analogs that are good inhibitors of [^{3}H] dihydroqueuine incorporation in tRNA induce the differentiation of murine erythroleukemia cells and human promyelocytic leukemia cells *in vitro*, where xanthine and uric acid that have little inhibitory capacity, cannot induce the differentiation of murine erythroleukemia cells (13).

Elliott, Katze and Trewyn investigated the effect of phorbol esters on early passage human cells using culture media that was supplemented with elevated levels of the four amino acids of queuine containing tRNAs. Human cells were treated with phorbol–12, 13–didecanoate at concentrations of 10^{-7} M and 10^{-8} M and were compared with controls. It was observed that a decrease of queuine content of tRNA was followed by an increase in saturation density. In later passages the queuine content of tRNA slowly increased to that found in control cells. This phenomenon was explained by a salvage mechanism that limits the exogenous queuine requirement of the cells, and the saturation density decreased compared to the control cells (14). In another publication that deals with cultured human fibroblasts, Elliott, Trewyn and Katze considered the possibility that phorbol esters, since they interact predominantly with membranes and membranes associated proteins, may inhibit queuine transport into the cell. They suggested an indirect action of phorbol esters since PDD inhibition was observed only 30 to 60 minutes after queuine was added to the system (15). In their data, they indicate that inhibition of queuine transport by the use of phorbol esters has as a result a decrease of queuosine content in tRNA. This conclusion also explained the observation that high levels of exogenous queuine can reverse the increase of saturation density that was observed with exposure to phorbol ester.

The function of queuosine in tRNA is not completely understood. Bienz *et*

al. had found that Eukaryotic G–$tRNA^{Tyr}$ can read amber codons, while Q–$tRNA^{Tyr}$ cannot in both a Xenopus oocyte protein synthesizing system and a reticulocyte cell–free protein synthesizing system (16). Shindo–Okada's explanation is that queuosine in tRNA prevents the miscoding of G in the third codon position (17). In contrast, Nogushi *et al.* has published that an *E. Coli* mutant that does not contain queuosine in its tRNA and lacks the enzyme tRNA–guanine ribosyltransferase did not show any amber suppressor activity. This mutant, however, was unable to grow on lactate under anaerobic conditions or when nitrate was added as an electron acceptor, suggesting that Q–tRNA has a specific role in the development of the nitrate reductase system in *E. Coli* (18).

There is significant evidence of queuine–lacking tRNA in tumor cells. Since, the *E. Coli* enzyme catalyzes an exchange of guanine into undermodified tRNA that contains guanine, but not into queuine containing tRNA, tRNA from different tumors were examined and incorporation of guanine was observed by the use of radioactive guanine. Incorporation of guanine was minimum for isolated tRNAs from normal tissues (1). That is, Morris hepatoma, SV40–transformed cells, hepatoma tRNA from cancer patients contain more G–tRNAs and therefore, can incorporate considerable amounts of guanine. In Murine Erythroleukemic cells Shindo–Okada mentions a decrease of G–tRNA when the cells differentiate and reach maturity (19). In contrast, this decrease of G–tRNA can be altered by the use of 12–O–tetradecanoylphorbol 13–acetate (TPA), that inhibits cell differentiation, demonstrating that G–tRNA is present in tumor cells and undifferentiated cells. Nishimura suggests that there is a limited amount of queuine in tumor cells and modification of tRNA with respect to queuine is not accomplished. Also, he proposes a factor present in tumor cells that is able to inhibit the insertion reaction, resulting in hypomodified Q–tRNA.

Protein Kinase C

Protein Kinase C is a serine and threonine specific protein kinase that is dependent upon calcium, phosphotidyl serine and diacylglycerol for activity. It has a crucial role in signal transduction for biologically active substances that activate cellular functions. This was first demonstrated when the enzyme's affinity for calcium was greatly increased in the presence of diacylglycerol, a breakdown product of inositol phospholipids (20). It is widely distributed in tissues and organs of mammals and other organisms. It has a single polypeptide chain that contains two functionally different domains. One domain is a hydrophobic domain that binds to membranes and the other domain is a hydrophilic domain containing the catalytically active center (21). Tumor promoters are not carcinogenic agents themselves but are able to enhance the development of tumors when used. Tumor promoting phorbol esters directly activate protein kinase C by substituting for diacylglycerol, however phorbol esters have a much longer biological half life than diacylglycerol. The phorbol ester 12–O–tetradecanoylphorbol–13–acetate (TPA) has a diacylglycerol–like structure, ester bonds at positions 12 and 13, and therefore replaces diacylglycerides at low but biologically active concentrations and increases the affinity of the enzyme for calcium by substituting for diacylglycerol. So, when phorbol ester tumor promoters bind to protein kinase C, they activate the enzyme and their affinity is equal to the activation constant (22, 23). Protein kinase C acts as a receptor of the tumor–promoting phorbol esters and activates cellular functions and proliferation by intercalating the phorbol esters into the membrane phospholipid bilayer. A number of other promoters that bind and activate protein kinase C have been identified such as mezerin, aplysiatoxin, debromoaplysiatoxin, and teleocidin (24). Chida *et al.* reported that TPA and teleocidin B cause a decrease in the activity of the cytosolic protein kinase C and a rapid activation of

protein kinase C in the plasma membranes. Most importantly, this decrease of cytosolic protein kinase C was equal to its increase in the membrane fraction (24). It has been discussed by Wooten *et al.*, that protein kinase C is mostly in an inactive form in the cytosol and when it is activated by biologically active substances, such as phorbol esters, it associates with cell membranes. This leads to the conclusion that protein kinase C activity is dependent on its environment and that phorbol esters are capable of modifying it (25).

It has been suggested that the expression of certain oncogenes, growth control related, are induced when protein kinase C is activated. Protein kinase C is associated with the plasma membrane and the cytoplasmic compartment in leukemia cells at the G_1 and S phase, where in the G_2 phase is primarily in the nucleus. The G_2 phase is actually the period that mRNA is being synthesized, suggesting a role in gene expression (26). The strongest evidence is the fact that nuclear RNA polymerase II is activated by protein kinase C and therefore, the enzyme responsible for synthesizing mRNA is a protein kinase C dependent enzyme. The activation of polymerase is shown by an increase of the initial rate of RNA synthesis and an increase of substrate–polymerase binding affinity which is proven by lower K_m values.

It has been reported by Elliott and Crane that modulation of protein kinase C activity is related to queuine uptake in human fibroblasts (27). In their experiments, protein kinase C was activated by addition of diolein, dicapryloyl–glycerol, phosphatidylserine and the calcium ionophore A23187. This activation of protein kinase C resulted in stimulation of rQT_3 uptake. Depression of rQT_3 was observed when protein kinase C was inhibited by H–7 (1–[5–isoquinoline sulfonyl] –2–methyl piperazine dihydrochloride) inhibitor that is known to directly interact with the active site of the enzyme. Other inhibitors of protein kinase C were used

(staurosporine and sphingosine) and it was concluded that protein kinase C activity regulates the queuine uptake efficiency in which case both enzymes are associated with tumor promotion.

Objectives of Study

Our project involved the isolation and purification of the enzymes tRNA–guanine ribosyltransferase and protein kinase C. It is known that the insertion enzyme inactivates rapidly and therefore is quite unstable. Since protein kinase C controlls rQT_3 uptake in fibroblasts, we attempted to see if protein kinase C could activate the insertion enzyme *in vitro* as well. This would demonstrate that tRNA–guanine ribosyltransferase is a protein kinase C dependent protein and its instability could be related to its degree of phosphorylation.

MATERIALS AND METHODS

Materials

Buffer A: 10 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 1.0 mM EDTA, 6 mM 2–mercaptoethanol, 10% glycerol, calpain inhibitor I 1 mg/ℓ, leupeptin 1 mg/ℓ, pepstatin A lyophilizate 1 mg/ℓ, chymostatin 1 mg/ℓ, and aprotinin 1 mg/ℓ.

Buffer B: 10 mM sodium phosphate (pH 6.9), 10 mM 2–mercaptoethanol, 10% glycerol, calpain inhibitor I 1 mg/ℓ, leupeptin 1 mg/ℓ, pepstatin A lyophilizate 1 mg/ℓ, chymostatin 1 mg/ℓ, and aprotinin 1 mg/ℓ.

Buffer C: 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 1.0 mM EDTA, 6mM 2–mercaptoethanol, and 50% glycerol.

Buffer D: 20 mM Tris–HCl (pH 7.5), 1.0 mM calcium chloride, 0.2 mM phenyl methyl sulfonyl fluoride (PMSF), and 50 mM 2–mercaptoethanol.

Buffer E: 20 mM Tris–HCl (pH 7.5), 5.0 mM EGTA, 2.0 mM EDTA, and 0.2 mM PMSF.

Buffer F: 20 mM Tris–HCl (pH 7.5), 2.0 mM EGTA, 2.0 mM EDTA, and 50 mM 2–mercaptoethanol.

Buffer G: 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol and 20 mM Tris–HCl (pH 7.5).

Buffer H (QRTase reaction buffer): 10 mM Tris–HCl (pH 8.0), 35 mM KCl, 1 mM magnesium chloride, and 5 mM 2–mercaptoethanol.

Buffer I (PKC reaction buffer): 100 mM Tris–HCl (pH 7.5), 35 mM KCl, 1 mM magnesium chloride, and 5 mM 2–mercaptoethanol.

Yeast Transfer RNA: purchased from Boehringer Manheim (West Germany), diluted to a concentration of 10 A_{260} stock solution,.and stored at –20 °C.

Histones: Type III histones from calf thymus were purchased from Sigma. They were diluted in water at a concentration of 16 mg/mℓ (10X solution), and were used to determine protein kinase C's activity.

Phospholipids: Protein kinase C activators are absolutely essential for all the assays where protein kinase C is present. Diolein and phosphatidylserine were purchased from Sigma. Diolein was diluted in acetone at a concentration of 200 mg/mℓ, and phosphatidylserine was diluted in chloroform at a concentration of 12 mg/mℓ. Those two were combined so that our final solution contained 20 mg of diolein and 18 mg of phosphatidylserine.

ATP: It was purchased from Fisher Scientific (Biotechnology grade), it was diluted in water at a final concentration of 1 mM solution, and it was used in protein kinase C assay.

7–Methylguanine: It was purchased from Sigma and it was diluted with water to make 100 μM stock solution.

Sphingosine: It was obtained from Biomol Research Laboratories of Plymouth Meeting PA. The final stock concentration was 100 μM in DMSO.

Staurosporine: It was purchased from Sigma and it was diluted with water to make 2×10^{-6} M stock solution.

H–7 (1–[5–isoquinoline sulfonyl]–2–methyl piperazine dihydrochloride): This protein kinase C antagonist was obtained from Seikagaku America, Inc. and was dissolved in water at a stock solution concentration of 100 μM.

[^{3}H]–Guanine: (1 mCi, 168 Ci/mmol, 0.5 mCi/ml) was purchased from Amersham Corp., Arlington Heights Ill. The concentration of the stock was

0.5 mCi/mℓ and was diluted with water at a final concentration of 10 μCi/mℓ.

gamma–[^{32}P]–ATP: It was obtained from Amersham in 250 μCi vials (3000 Ci/mmol).

Methods

Isolation and Purification of tRNA–Guanine Ribosyltransferase

We modified the published procedure by Okada *et al.* as follows: three rat livers were removed and homogenized in buffer A (see materials for all buffers). Centrifugation followed at 10,000 x g for 20 minutes at 4 °C. The supernatant was centrifuged at 100,000 x g for 2 hours at 4 °C. The supernatant was diluted three times with buffer A and was ready to be loaded on a 4 x 17 DEAE–52 column which was equilibrated with 300 mℓ buffer A. The column was eluted with a linear gradient of 0 – 0.6 M NaCl in buffer A. Active fractions were collected, combined and dialyzed against buffer B for two hours. The active fractions that had been collected were loaded onto a phosphocellulose P11 column (2.6 x 12) which had been pre–equilibrated with buffer B and washed. The column was eluted with 200 mℓ linear gradient of 0 – 1.0 M NaCl in buffer B. The active fractions were combined and dialyzed for 2 hours against buffer C. The enzyme preparation was stored at –70 °C.

Isolation and Purification of Protein Kinase C

Twenty rat brains were obtained (by asphyxiating with CO_2 twenty rats) and immediately placed in buffer D at 4 °C. Manual homogenization followed in a minimal amount of buffer (60 mℓ) and removal of debris was accomplished by centrifugation at 1000 x g at 4 °C. Ultracentrifugation followed at 100,000 x g for 1 hour at 4 °C. The pellet was suspended in buffer E and disrupted by sonic oscillation (three times for 30 seconds) using the Branson Sonifier Cell Disrupter

185. The sonicate was stirred in an ice bath for 1 hour, then it was centrifugated at 100,000 x g for 60 minutes, at 4 °C. The supernatant was treated with ammonium sulfate to reach 21% wt/vol solution, stirring for 30 minutes, resting for 30 minutes in an ice bath, and finally centrifuged for 15 minutes at 15,000 x g. The ammonium sulfate concentration was then increased to 45% wt/vol solution in the supernatant, stirring, resting and centrifugation was repeated as before. The pellet was resuspended in buffer F and dialyzed for 4 hours. A DEAE–52 (4 x 17 cm) column was equilibrated and washed with buffer F and the enzyme was eluted with a step gradient of 30 ml each of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 M NaCl in buffer F. The absorbance of eluted material was monitored at 280 nm wavelength and the activity of the collected fractions (2 mℓ) was tested by running a gamma–[^{32}P]–ATP assay. The active fractions were concentrated with an Amicon filter unit using a YM5 filter.

The concentrated enzyme was further fractionated by using a Mono Q (HR 10/10) column with a high resolution FPLC system (LKB system 2150). The column was equilibrated with buffer G and after the enzyme was loaded, it was washed with the same buffer. A linear gradient of 0 – 300 mM NaCl in buffer G was used to elute Protein Kinase C which was again tested for activity and for protein concentration. All FPLC steps were performed at room temperature.

Activity Test for QRTase

Based on the fact that the insertion enzyme will not only irreversibly insert queuine in exchange for guanine but will reversibly exchange guanine for guanine, tritiated guanine was used to determine the active fractions after isolation and purification. QRTase buffer H, 0.2 A_{260} yeast tRNA, 1 x 10^{-4} M ^{3}H–guanine (1 μCi in each assay tube), 100 μl of enzyme fraction and water in a total amount of 300 μl were combined and incubated at 37 °C for 45 minutes. The reaction was

stopped with 1/3 volume of 30% trichloroacetic acid, kept on ice for 30 minutes and the radioactive acid precipitate was collected on glass fiber filters and analyzed using the Beckman LS 5000 CE scintillation counter.

Determination of QRTase's Maximum Activity

In order to determine the concentration of QRTase where maximum activity occurs, the same test as above was used varying the volume of the enzyme from 20 to 120 $\mu\ell$.

Activity Test for Protein Kinase C

Protein Kinase C is responsible for phosphorylating histone proteins. In order to determine the activity of the enzyme gamma–[^{32}P]–ATP was purchased and used in our assay. Protein Kinase C reaction buffer I, 1 μCi gamma–[^{32}P] –ATP, 1 mM calcium chloride, phospholipids (54 μg diolein,48 μg phosphatidyl–serine), 0.48 mg histone, the enzyme and water up to a total volume of 300 $\mu\ell$ were combined and tested for activity. A separate assay was performed to determine the concentration of the enzyme where maximum activity is seen.

Rat Brain PKC Assay Including Modulators

This assay was as outlined for the activity test of protein kinase C with the addition of the three known inhibitors of the phosphorylating enzyme, 100 μM spingosine, 0.2 μM staurosporine and 100 μM H–7. The controls included protein kinase C with all its activators and the phosphorylating enzyme in the absence of histone proteins.

QRTase and Protein Kinase C Combined Assay

In this experiment both enzymes were used to ascertain an increase in activity and therefore, examine the dependence of the insertion enzyme on the phosphorylating enzyme. Protein Kinase C reaction buffer I, calcium chloride (1 mM), the activators diolein (54 μg) and phosphatidylserine (48 μg), tritiated

guanine (20 mCi), 1 mM ATP, tRNA of 0.2 Absorbance at 260 nm, 60 $\mu\ell$ protein kinase C, 100 $\mu\ell$ QRTase, and water for a total volume of 300 $\mu\ell$, were combined, incubated at 37 °C for 30 minutes. The reaction was stopped with 100 $\mu\ell$ of 30% trichloroacetic acid, kept on ice for 30 minutes and the radioactive acid precipitate was collected on glass fiber filters and analyzed using the Beckman LS 5000 CE scintillation counter. Appropriate controls were included.

QRTase and Protein Kinase C Combined Assay Including Modulators

The same assay as above was performed, only this time different modulators were included. A stock solution of 100 μM of 7–methyl guanine was prepared and was used also in this assay at a final concentration of 5 μM as negative control. Sphingosine, staurosporine and H–7 all known inhibitors of Protein Kinase C were included at a final concentration of 100 μM, 0.2 μM and 100 μM respectively.

^{32}P Incorporation into QRTase by PKC

This test was performed to check if ^{32}P is incorporated into the insertion enzyme in the presence of the phosphorylating enzyme. The controls were protein kinase C in the presence and absence of histone proteins. A separate tube contained the insertion enzyme instead of the histone proteins. Incubation took place for 45 minutes. The controls were precipitated with 300 mℓ of 30% trichloroacetic acid, while the reaction mixture that contained the insertion enzyme was transfered on filters (in fiber glass), in 20 $\mu\ell$, 40 $\mu\ell$ and 60$\mu\ell$ quantity, and precipitation occured dropwise in the fiber glass. Then the filters were transfered under vacuum, further precipitating them with 15 mℓ of 5% trichloroacetic acid and drying the filters with 95% ethanol.

Bio–Rad Protein Assay

The Bio–rad protein assay was chosen over the Lowry method because of the stability of the dye–protein complex that is formed, absence of interferences and

absence of critical timing compared to the Lowry assay. Accurate quantitation of protein is accomplished by constructing a standard curve, using bovine gamma globulin protein. Absorbance was measured at 595 nm. The unknown protein concentration was read out of the standard curve.

Calculations

The mean, $\bar{x}$, was evaluated by using the following formula:

$$\bar{x} = \frac{\sum_{1}^{N} x_i}{N}$$

where N is the number of data points and x_i is the value of the ith data point. Standard deviation was applied to the data depicted in figures 13 and 14 utilizing the following expression:

$$S = \left[\frac{\sum_{1}^{N} (x_i - \bar{x})^2}{(N-1)} \right]^{1/2}$$

where N = 3 is the number of samples used and x_i is the value of each sample.

The specific activity of tRNA–guanine ribosyltransferase was calculated based on the following formula:

$$pmoles\ of\ ^{3}H\text{–}G/hour\ of\ incubation/\mu g\ of\ tRNA/mg\ of\ protein.$$

Similarly, the specific activity of protein kinase C was determined as follows:

$$pmoles\ of\ ^{32}P\ incorporated/mg\ of\ histone/mg\ of\ protein.$$

RESULTS

Isolation and Purification of tRNA–Guanine Ribosyltransferase

Several attempts were made to isolate and purify the insertion enzyme. We found it difficult to isolate when a large number of rat livers were used (50 rat livers). It was surmised that since the procedure took a longer time, the enzyme was inactivated, and we were unable to identify any active fractions. When a phosphatase substrate (p–nitrophenyl phosphate) was added to our buffers, we obtained yellow fractions in the purification protocol. This indicated that there was significant phosphatase activity in our protein fractions. Phosphatase activity could negatively effect our enzyme if it is a target for protein kinase C. We also believe that the protease inhibitors (calpain inhibitor I, leupeptin, pepstatin A, chymostatin and aprotinin), which were added to our buffers, were essential to retain an active enzyme. Figure 2 demonstrates the activity profile of DEAE–52 column. The activity of the combined fractions after the DEAE–52 isolation is shown in Figure 3. During the phosphocellulose (P11) purification (activity profile shown in Figure 4) we excluded the protease inhibitors from the elution buffer and the dialysis time was shortened from 16 hours to 2 hours in the last step of the procedure. The activity of the combined fractions is demonstrated in Figure 5. A Bio–Rad assay was performed after isolation and purification of the enzyme. Standard curves were constructed and the protein concentration of the active fractions after DEAE–52 and phosphocellulose columns were read from this curve. The following table

Figure 2. Activity profile of rat liver QRTase during DEAE–52 isolation. Fractions 14, 15, and 16 were collected and combined.

Activity Profile of Rat Liver QRTase during DEAE isolation.

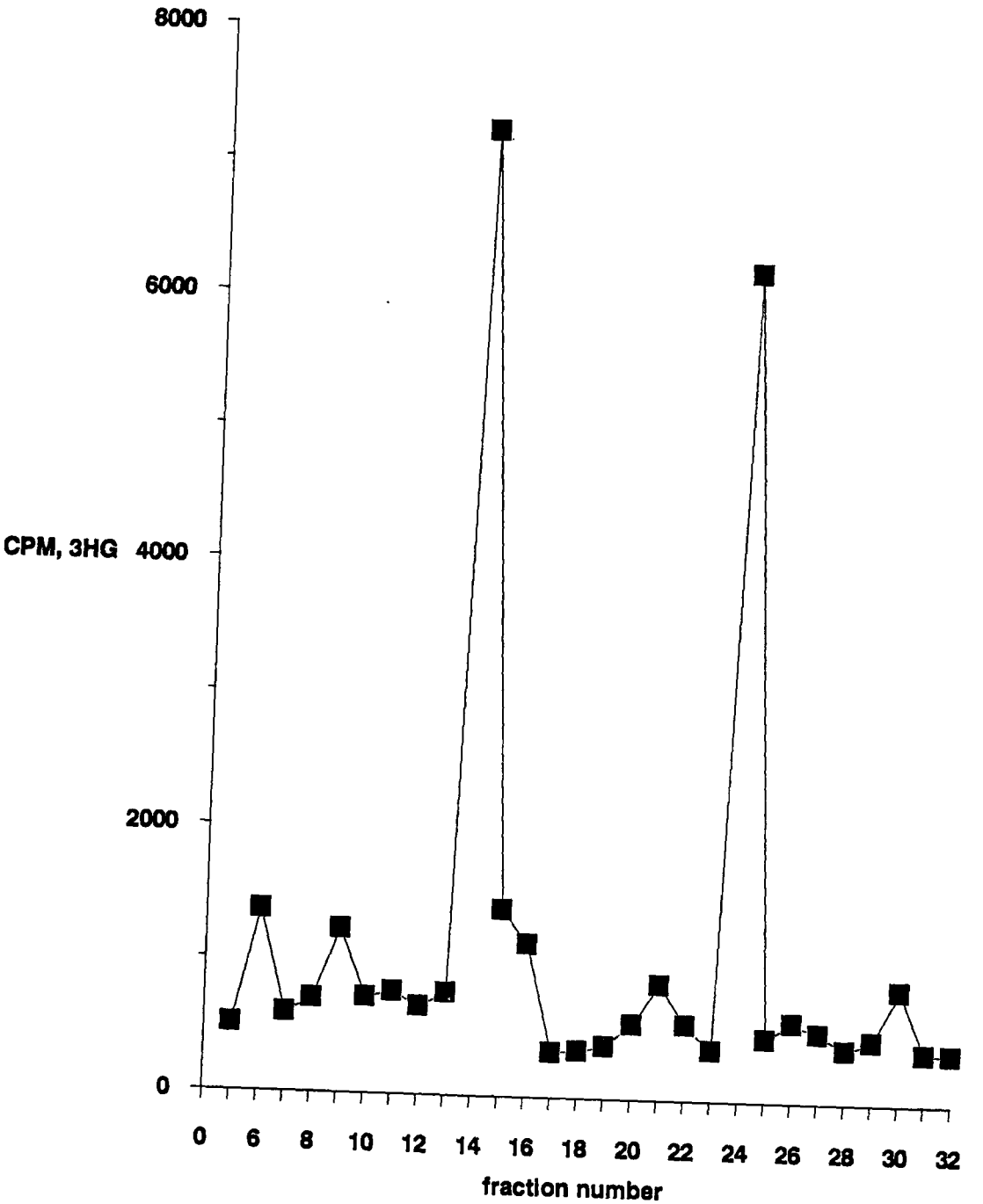


Figure 3. Combined fractions of rat liver QRTase assay after DEAE–52 isolation.

Combined Fractions of Rat Liver QRTase Assay after DEAE Isolation.

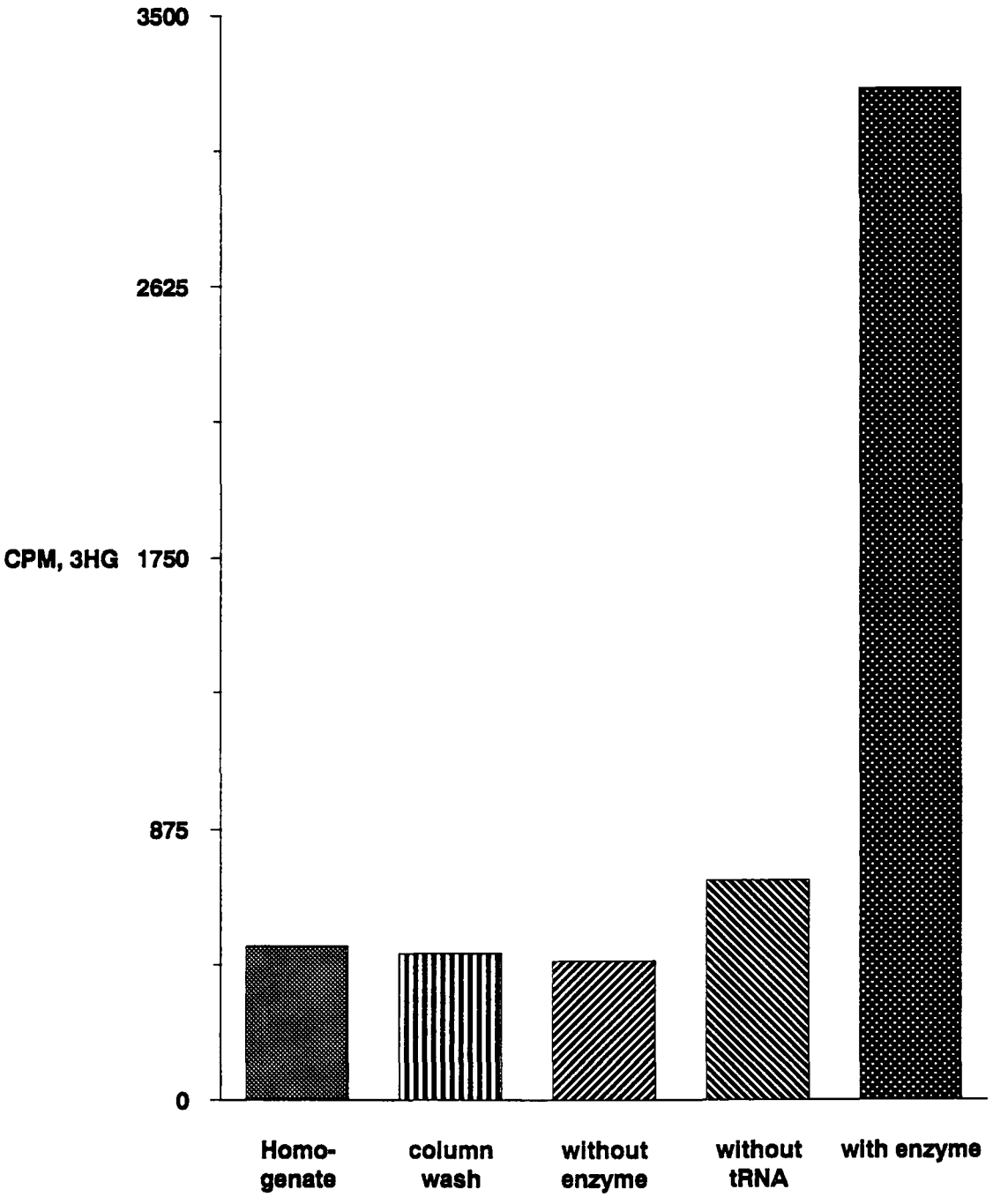


Figure 4. Activity profile of rat liver QRTase during phosphocellulose isolation. Two fractions (23, 24) of 2 mℓ each were collected.

Activity Profile of Rat Liver QRTase during Phosphocellulose Isolation.

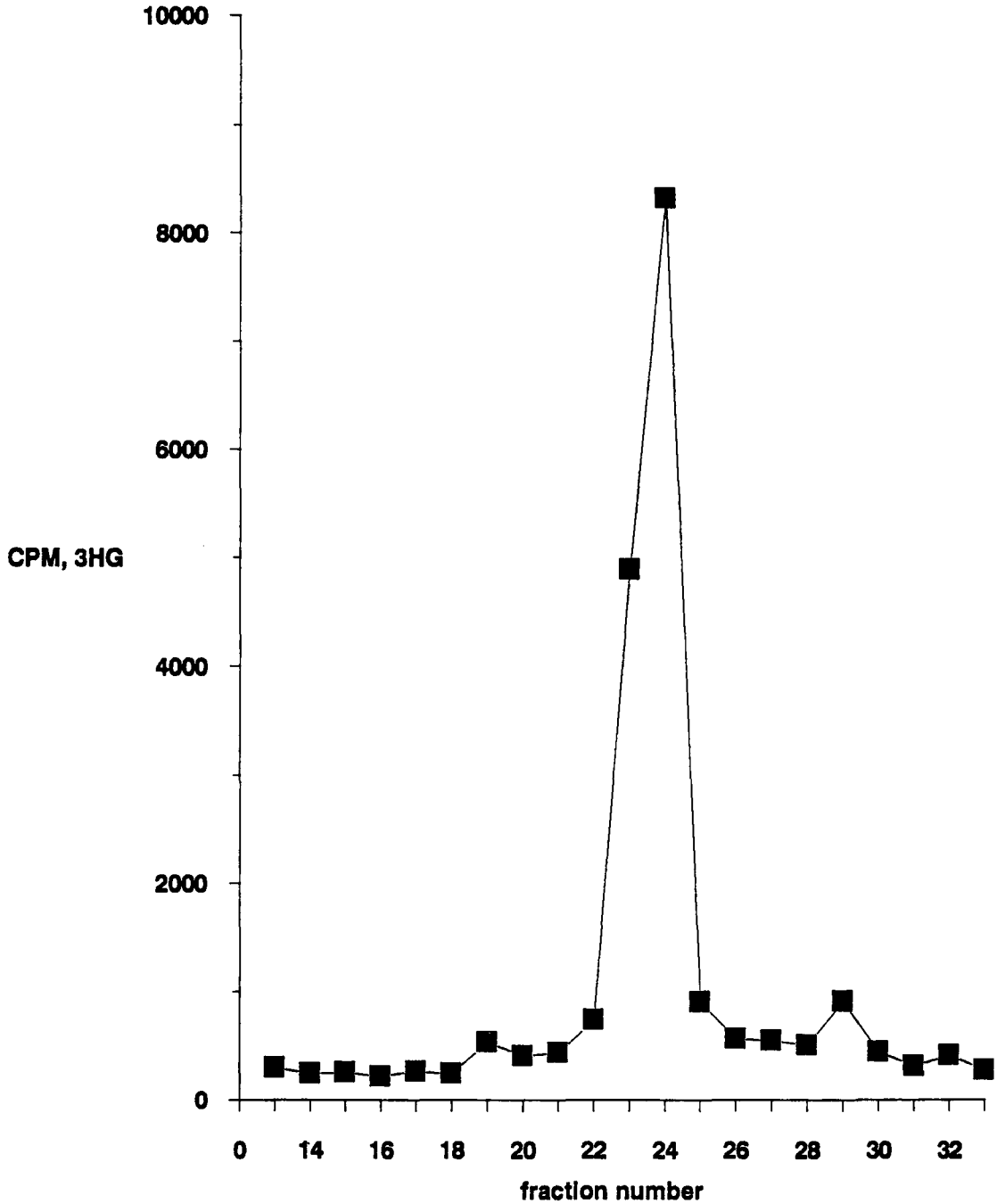
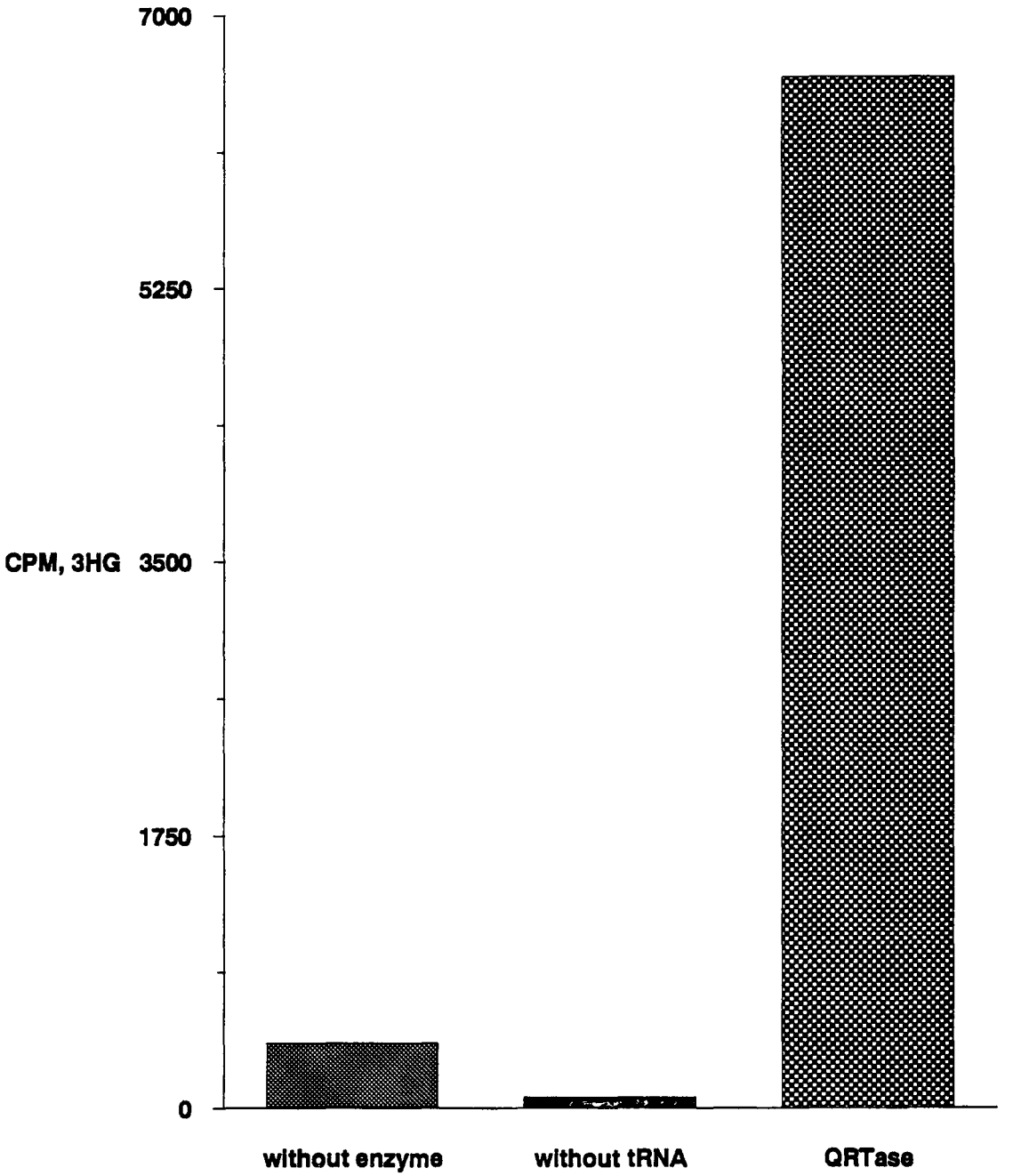


Figure 5. Combined fractions of rat liver QRTase assay after phosphocellulose isolation.

Combined Fractions of Rat Liver QRTase Assay after Phosphocellulose Isolation.



(Table 1) indicates the protein concentration and the specific activity of the insertion enzyme.

Table 1: Summary of Rat Liver tRNA–Guanine Ribosyltransferase.

fraction	total protein (mg)	total units (CPM)	specific activity	vol (mℓ)
(I) 3 rat livers supernatant	286.0	499.01	0.0005	60
(II) DEAE–52 column chromatography	6.1	3268.17	0.1674	5
(III) P–cellulose column chromatography	1.0	6609.54	2.0655	4

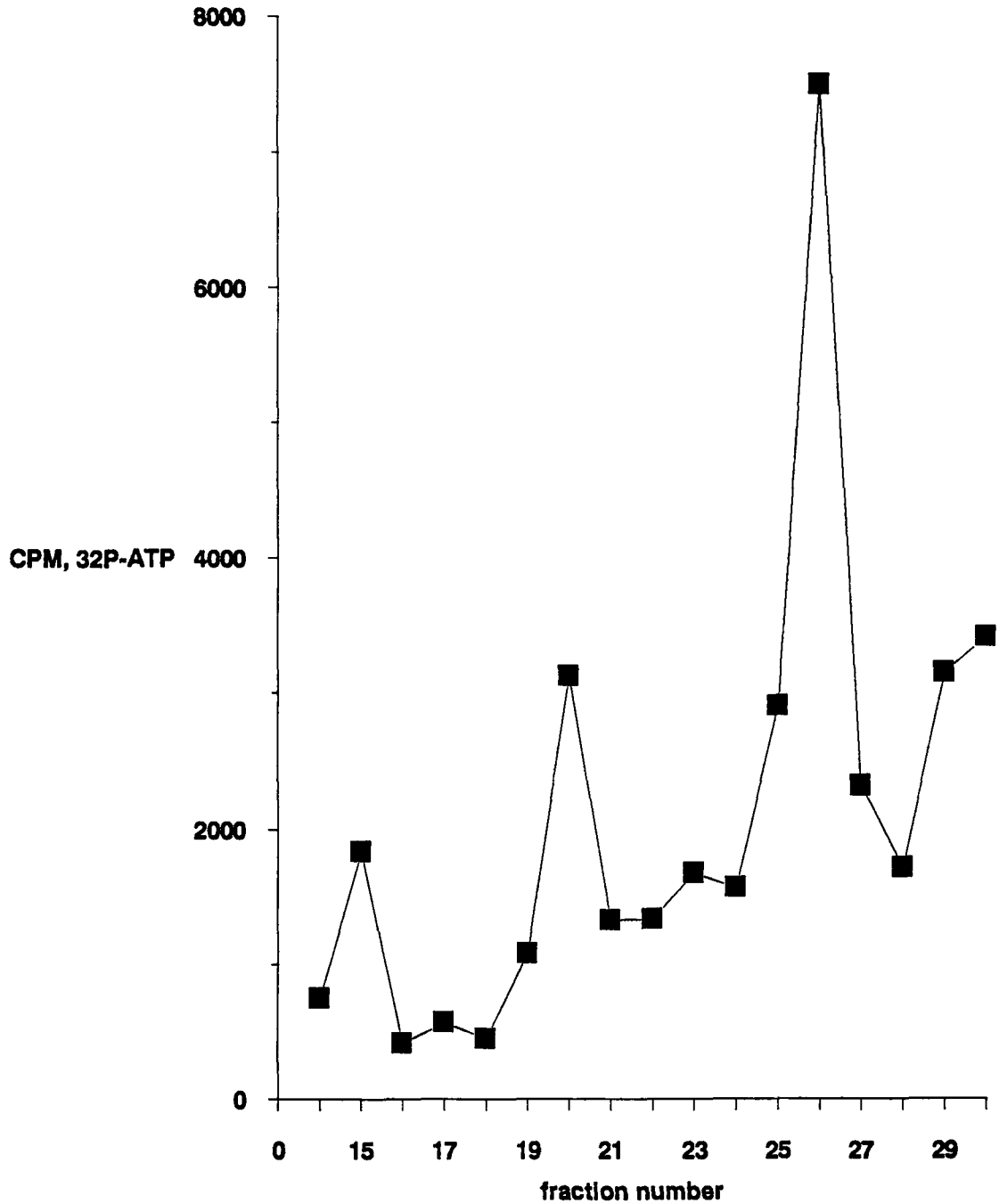
The DEAE–52 column chromatography and phosphocellulose (P11) were effective, resulting in 4131 fold purification of the insertion enzyme.

Isolation and Purification of Protein Kinase C

The rat brains that were collected were frozen with liquid nitrogen and were stored at –70 °C for four weeks before we attempted to isolate the enzyme. The procedure was followed as described in the methods section and the active fractions were easily identified using the gamma–[^{32}P]–ATP assay. All steps took place at 4 °C except for the last fractionation, using the Mono Q column, which was performed at room temperature. Even though this took place at 25 °C, the stability of the enzyme was such that we were able to identify and combine the active fractions. The activity profile of the DEAE–52 column (Figure 6) shows that six

Figure 6. Activity profile of rat brain protein kinase C during DEAE–52 isolation. Six fractions (25, 26, 27, 28, 29, 30) of 3.6 mℓ each were collected and combined.

Activity Profile of Rat Brain PKC during DEAE Isolation.



fractions of 3.6 mℓ each were identified as the ones that contained the enzyme and Figure 7 demonstrates the combined active fractions. These were concentrated utilizing an Amicon filtration unit. Further purification by the use of Mono Q column (activity profile shown in Figure 8) resulted in five active fractions of 2 mℓ each with an average activity of 7.13 pmol ATP/mg histone/mg protein (Figure 9). Table 2 summarizes the yield, specific activity and the extent of purification of the enzyme.

Table 2: Summary of Purification of Rat Brain Protein Kinase C.

fraction	total protein (mg)	total units (CPM)	specific activity	vol (mℓ)
(I) 20 rat brains homogenate	50.0	455.00	0.0342	180.0
(II) DEAE–52 column chromatography	5.7	3504.00	2.3076	21.6
(III) Mono Q (HR 10/10) column chromatography	3.5	6651.80	7.1341	10.0

As it is indicated, 180 mℓ of 20 rat brain homogenate was purified achieving a 208.6 fold purification of the phosphorylating enzyme.

Combined Assay of Rat Liver QRTase and Rat Brain PKC

The insertion enzyme lost its activity several days after it was isolated, purified and stored at –70 °C as it is shown in Figure 10. A combined assay was performed using QRTase and protein kinase C to determine whether tRNA–guanine

Figure 7. Combined fractions of rat brain protein kinase C after DEAE–52 isolation.

Combined Fractions of Rat Brain PKC Assay after DEAE Isolation.

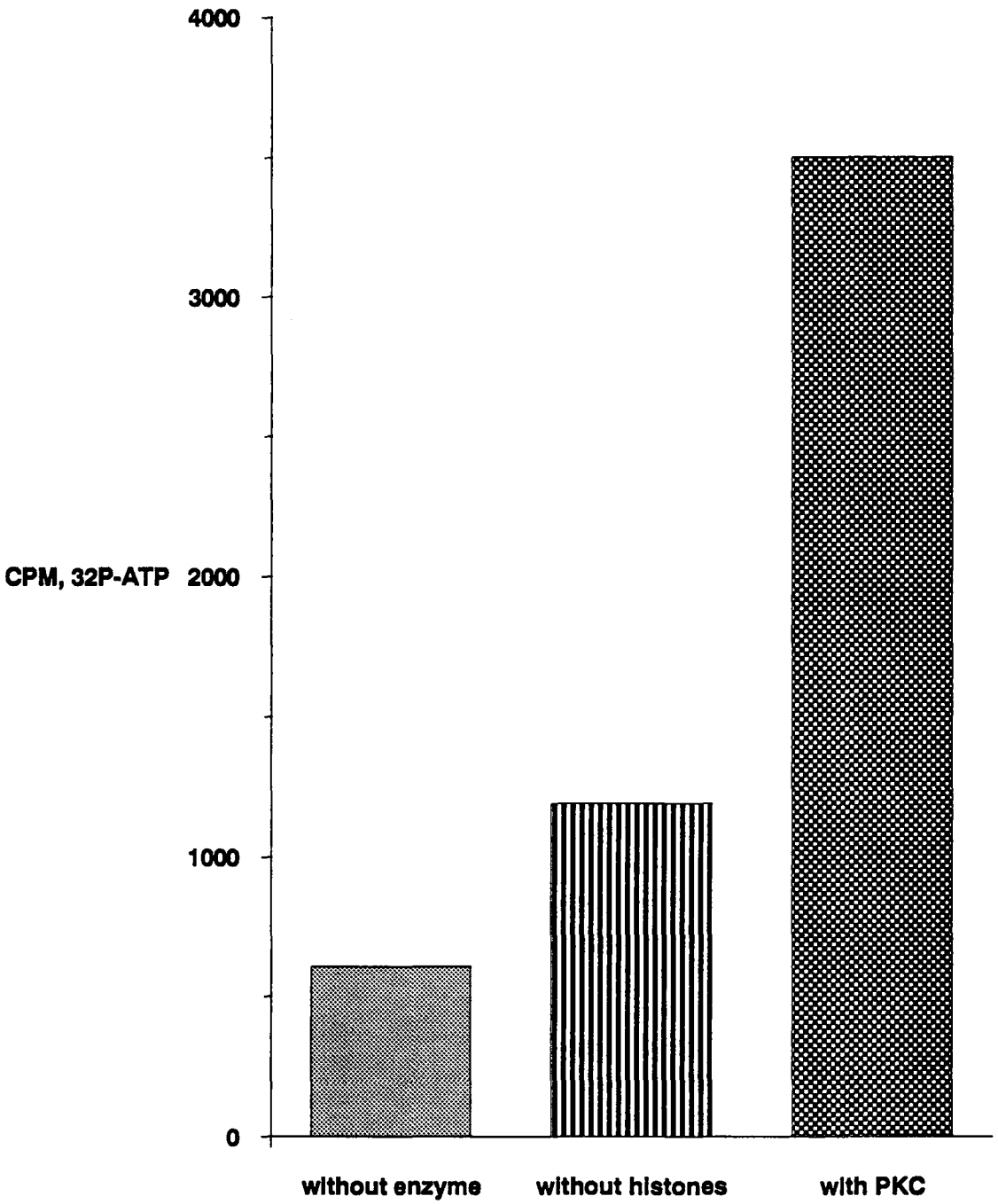


Figure 8. Activity profile of rat brain protein kinase C during Mono Q purification. Five fractions of 2 mℓ each were collected and 50% glycerol was added before storage. Fraction number 34 was not combined with fractions 31, 32, 33, 35, and 36 because of its low counts.

Activity Profile of Rat Brain PKC during Mono Q Purification.

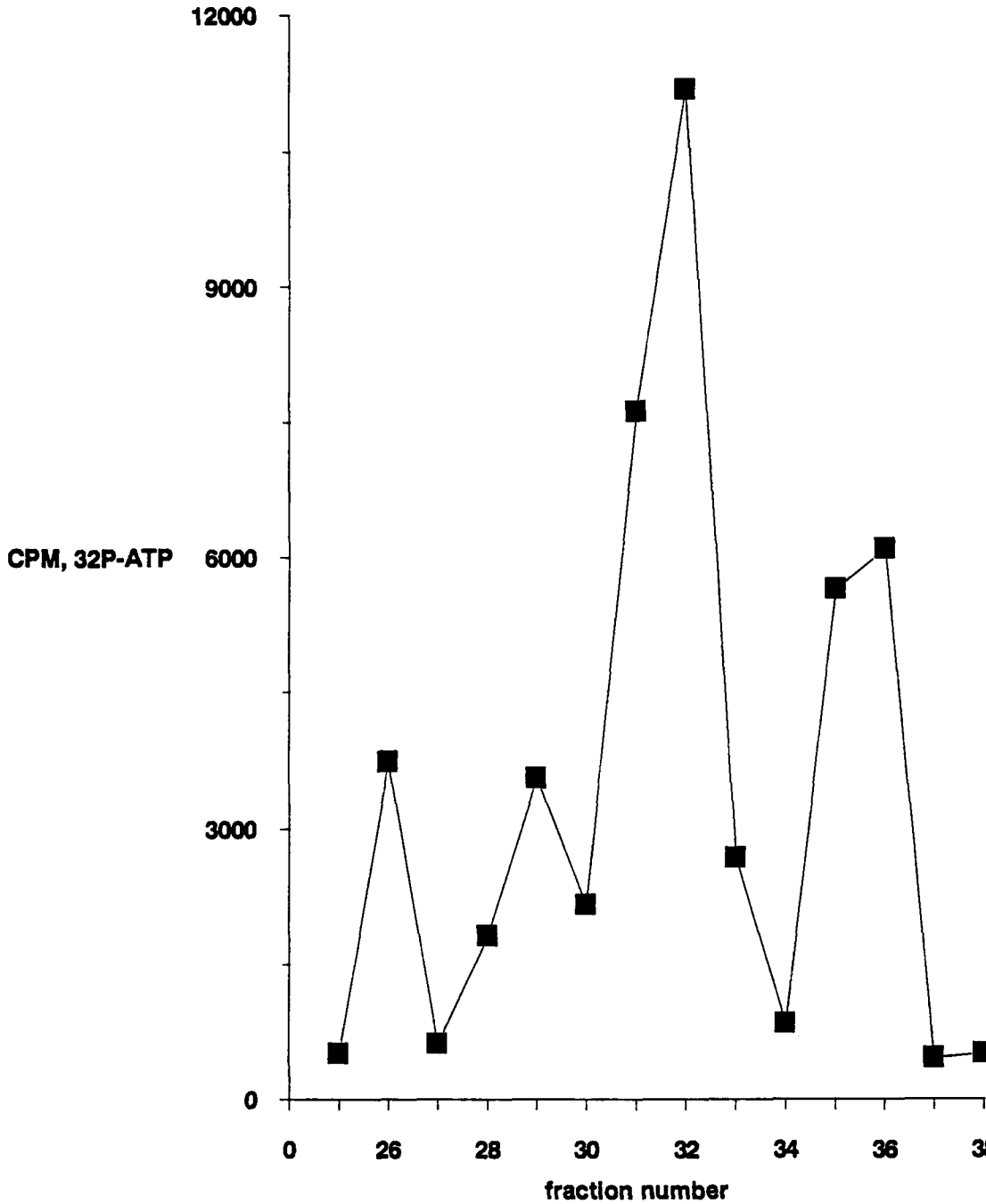


Figure 9. Combined fractions of rat brain protein kinase C assay after Mono Q column.

Combined Fractions of Rat Brain PKC Assay after Mono Q Column.

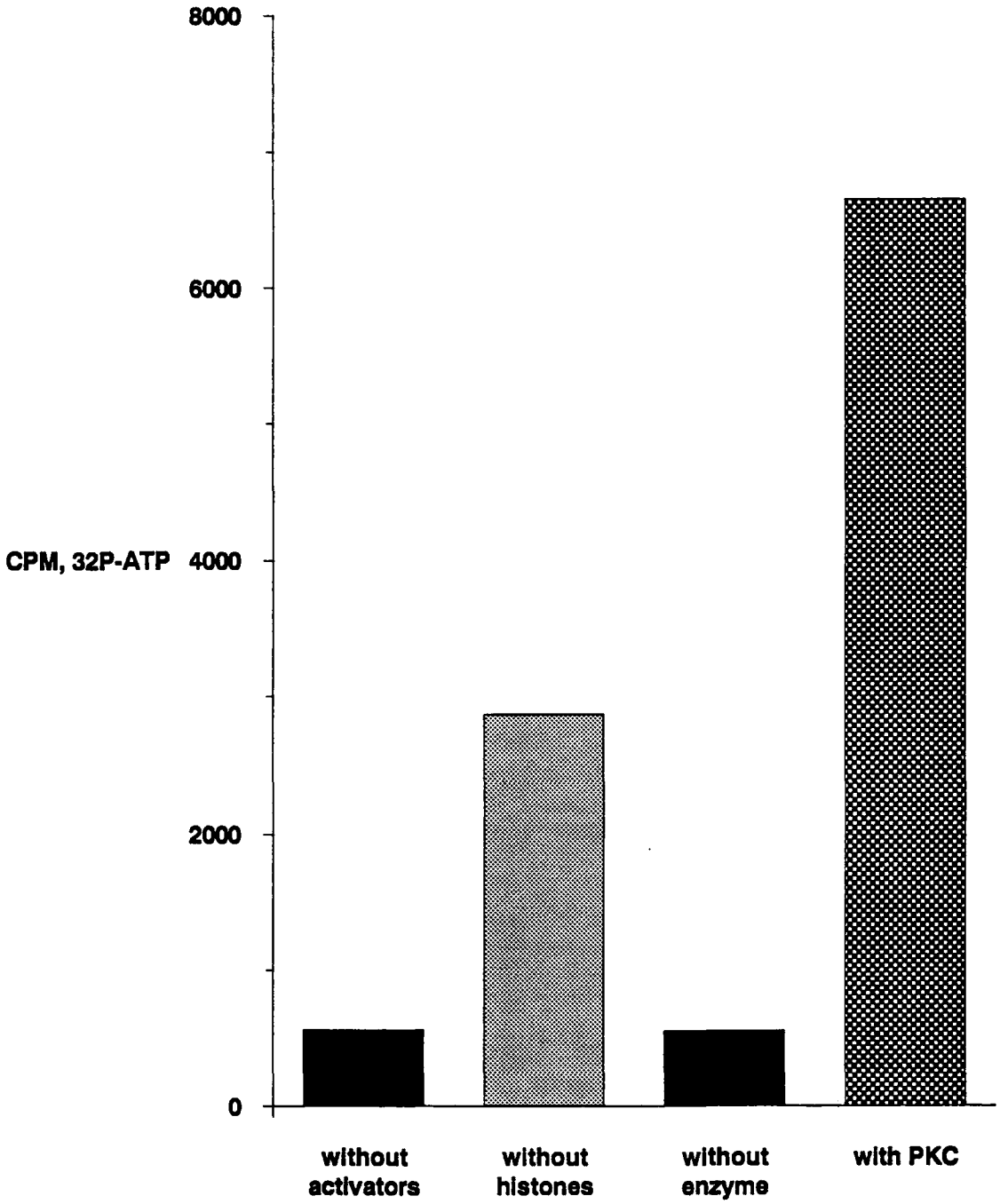
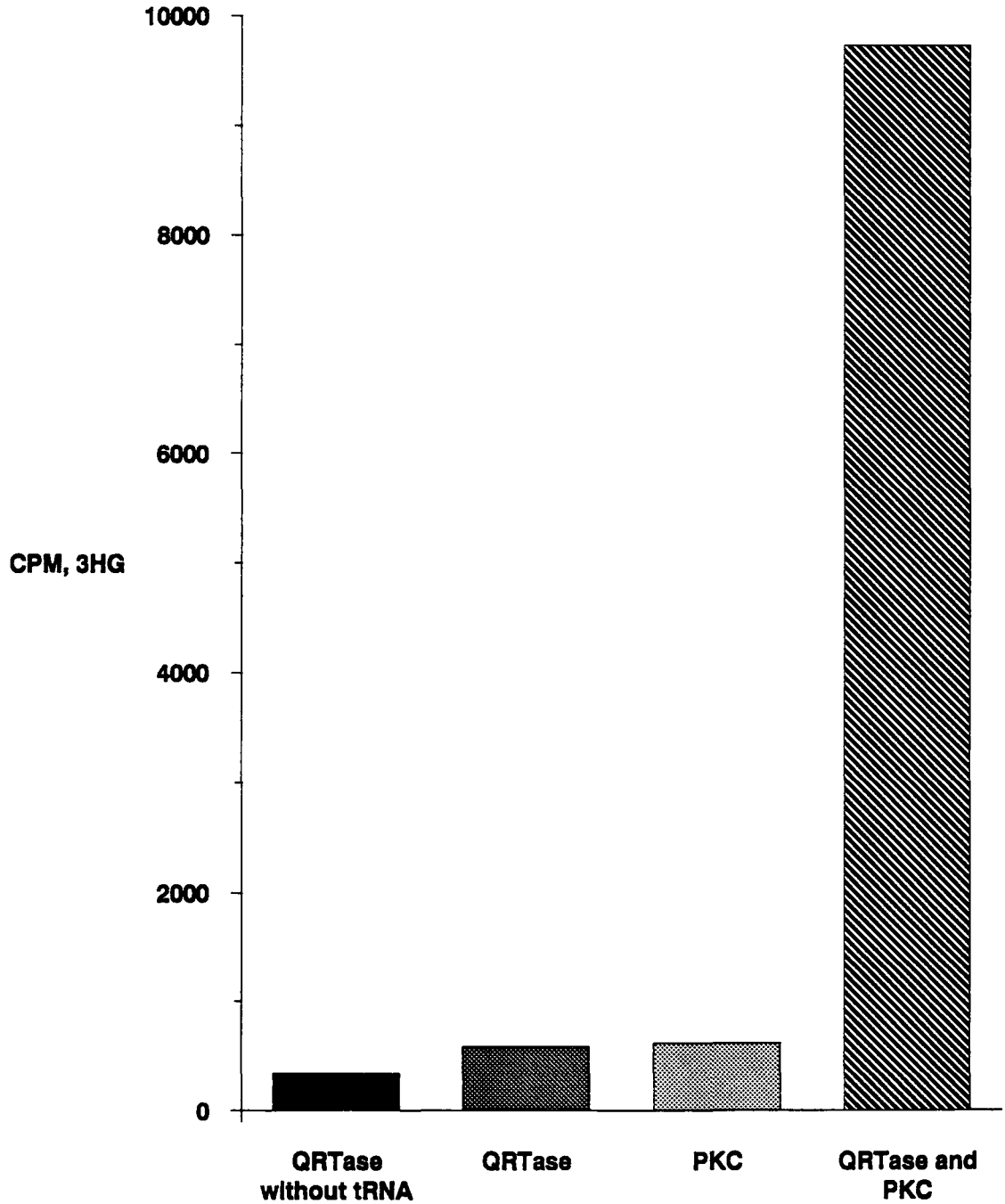


Figure 10. Combined assay of rat liver QRTase and rat brain protein kinase C. QRTase is shown to be inactive. Protein kinase C was tested for insertion. Reactivation occurs when protein kinase C with all its activators is added to tRNA–guanine ribosyltransferase.

Combined Assay of Rat Liver QRTase and Rat Brain PKC.



ribosyltransferase regains activity through the phosphorylating enzyme. We tested the activity of QRTase. As a control, the insertion enzyme without tRNA was assayed. We also included protein kinase C to assure there was no latent insertion activity and we examined the activity of the insertion enzyme in the presence of protein kinase C. The insertion enzyme resulted in an average of 0.19 pmol [^{3}H]–G/hour/μg tRNA/mg protein and 2.99 pmol [^{3}H]–G/hour/μg tRNA/mg protein when protein kinase C was included in the assay. It is important to note that when the insertion enzyme was tested after the phosphocellulose isolation the active fraction indicated 2.07 pmol ^{3}H–G/hour/μg tRNA/mg protein. This strongly suggests that the insertion enzyme was reactivated.

Determination of QRTase Maximum Activity

The maximum activity of the insertion enzyme was determined by varying the volume from 20 $\mu\ell$ to 120 $\mu\ell$ of the enzyme and, as it is shown on Figure 11, 100 $\mu\ell$ were needed to achieve it.

Determination of PKC Maximum Activity

Figure 12 demonstrates that the maximum activity of the phosphorylating enzyme is achieved when 60 $\mu\ell$ are used per assay tube. This test was performed several times in order to determine its maximum activity. We observed various protein kinase C activity in individual tubes stored at –70 ° C. This could have resulted from an inhomogeneous distribution of the enzyme in the glycerol media.

Rat Brain PKC Assay Including Modulators

Even though the phosphorylating enzyme was stored for eight months, at –70 ° C, it was still active when it was assayed with ATP and histones. Protein

Figure 11. Determination of QRTase maximum activity. This was demonstrated when 100 $\mu\ell$ of the insertion enzyme was used.

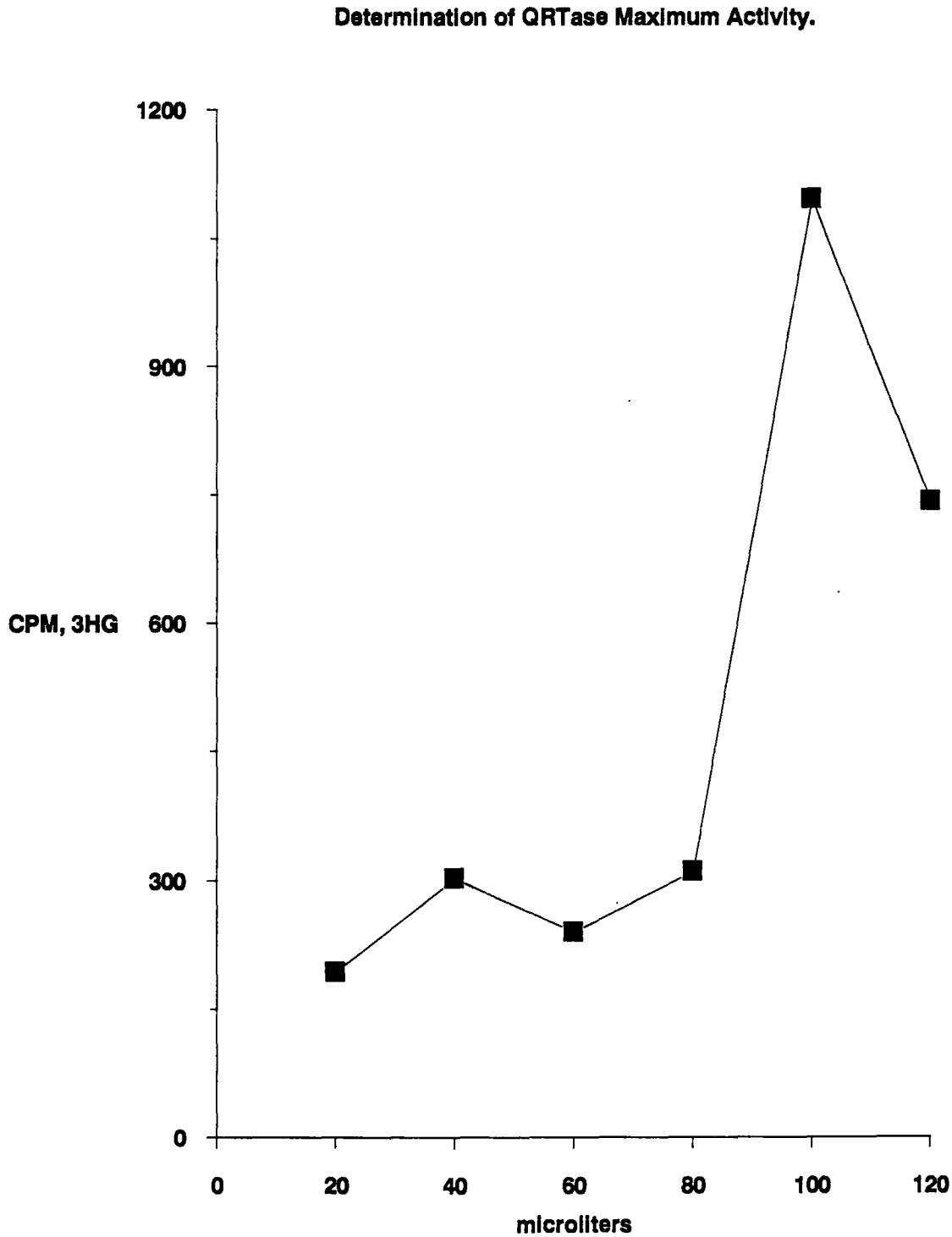
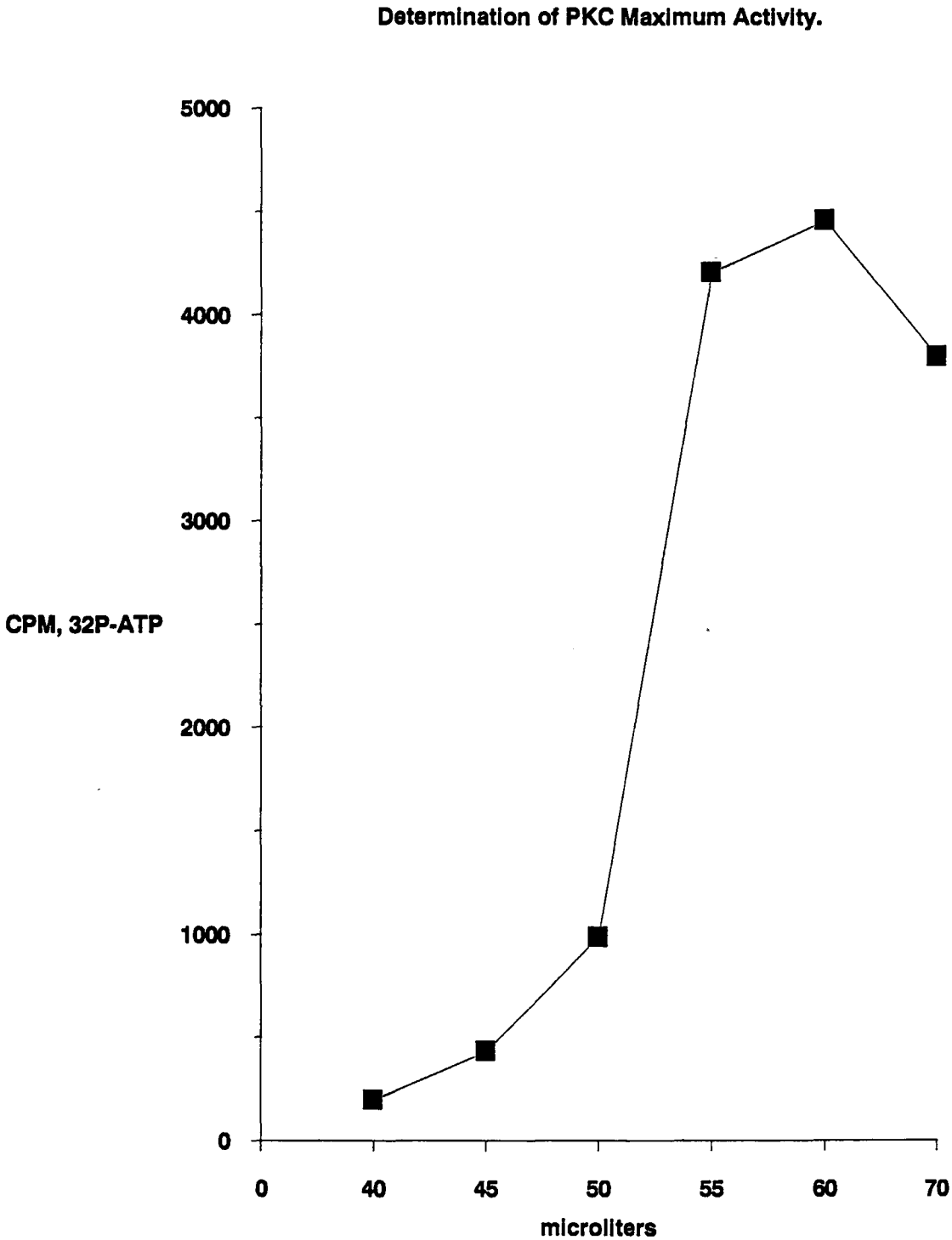

Determination of QRTase Maximum Activity.
1200
900
600
300
0
CPM, 3HG
0
20
40
60
80
100
120
microliters

Figure 12. Determination of protein kinase C maximum activity. This was demonstrated with 60 $\mu\ell$ of the phosphorylating enzyme.


Determination of PKC Maximum Activity.
5000
4000
3000
2000
1000
0
CPM, 32P-ATP
0
40
45
50
55
60
70
microliters

kinase C was then assayed to determine whether deactivation occurs when PKC inhibitors were added to the reaction mixture. 100 μM sphingosine, 0.2 μM staurosporine and 100 μM H–7 were used to check if the enzyme becomes inactive. Figure 13 demonstrates the deactivation of the enzyme when PKC inhibitors were used, showing maximum inhibition with 0.2 μM staurosporine. As indicated in the figure, PKC was tested for activity using as control, the enzyme with all its activators, excluding the histone proteins. From this figure it can be seen that minimum phosphorylation occurs in the absence of histone proteins.

Combined Assay of Rat Liver QRTase and Rat Brain PKC Including Inhibitors

The combined assay was repeated using the inhibitors 7–methylguanine (a known inhibitor of QRTase), sphingosine, staurosporine and H–7 (known inhibitors of protein kinase C). As shown in Figure 14, 5 μM of 7–methylguanine, 100 μM of sphingosine, 0.2 μM of staurosporine and 100 μM of H–7 were able to inhibit the activation of the insertion enzyme either by inhibiting the phosphorylating enzyme or inactivating the insertion enzyme.

^{32}P Incorporation into QRTase by Protein Kinase C

Figure 15 demonstrates the incorporation of ^{32}P into the insertion enzyme fraction when protein kinase C is present. This test was performed to actually determine if we could tag the insertion enzyme. As can be seen from the graph increasing amounts of tRNA–guanine ribosyltransferase result in greater incorporation of ^{32}P suggesting that the insertion enzyme is actually a protein kinase C dependent protein. Twenty microliters precipitated reaction mixture resulted in an average of 19.48 pmol/mg protein, fourty microliters yielded 27.14 pmol/mg protein and sixty microliters 31.41 pmol/mg protein.

Figure 13. Known inhibitors of protein kinase C were tested to determine whether they inhibit the phosphorylating enzyme: 100 μM sphingosine, 0.2 μM staurosporine and 100 μM H–7. Maximum inhibition occured with 0.2 μM staurosporine. The standard deviation for PKC is ±650.75, for the control (without histones) is ±38.16, for sphingosine is ±110.75, for staurosporine is ±16.76, and for H–7 is ±296.53.

Rat Brain PKC Assay Including Modulators.

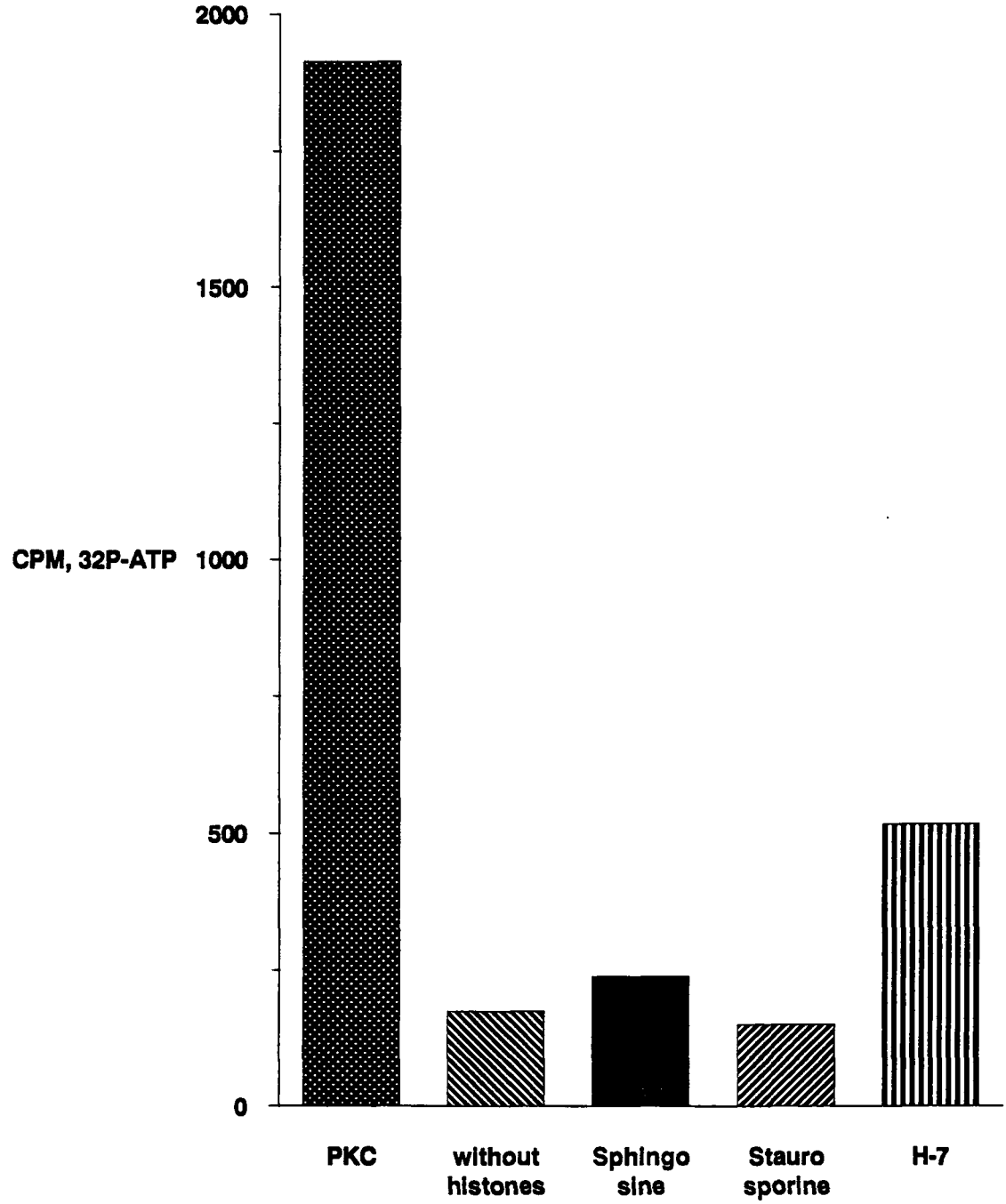


Figure 14. Combined assay of rat liver QRTase and rat brain protein kinase C in the precence of the inhibitors. Sphingosine, staurosporine and H–7 inhibited protein kinase C which in turn resulted in inability to reactivate the insertion enzyme. 7–methylguanine, a known inhibitor of QRTase, was also tested. The standard deviation for 7–methylguanine is ±211.43, for sphingosine is ±20.22, for staurosporine is ±225.66, and for H–7 is ±72.55.

Combined Assay of Rat Liver QRTase, Rat Brain PKC, and Modulators.

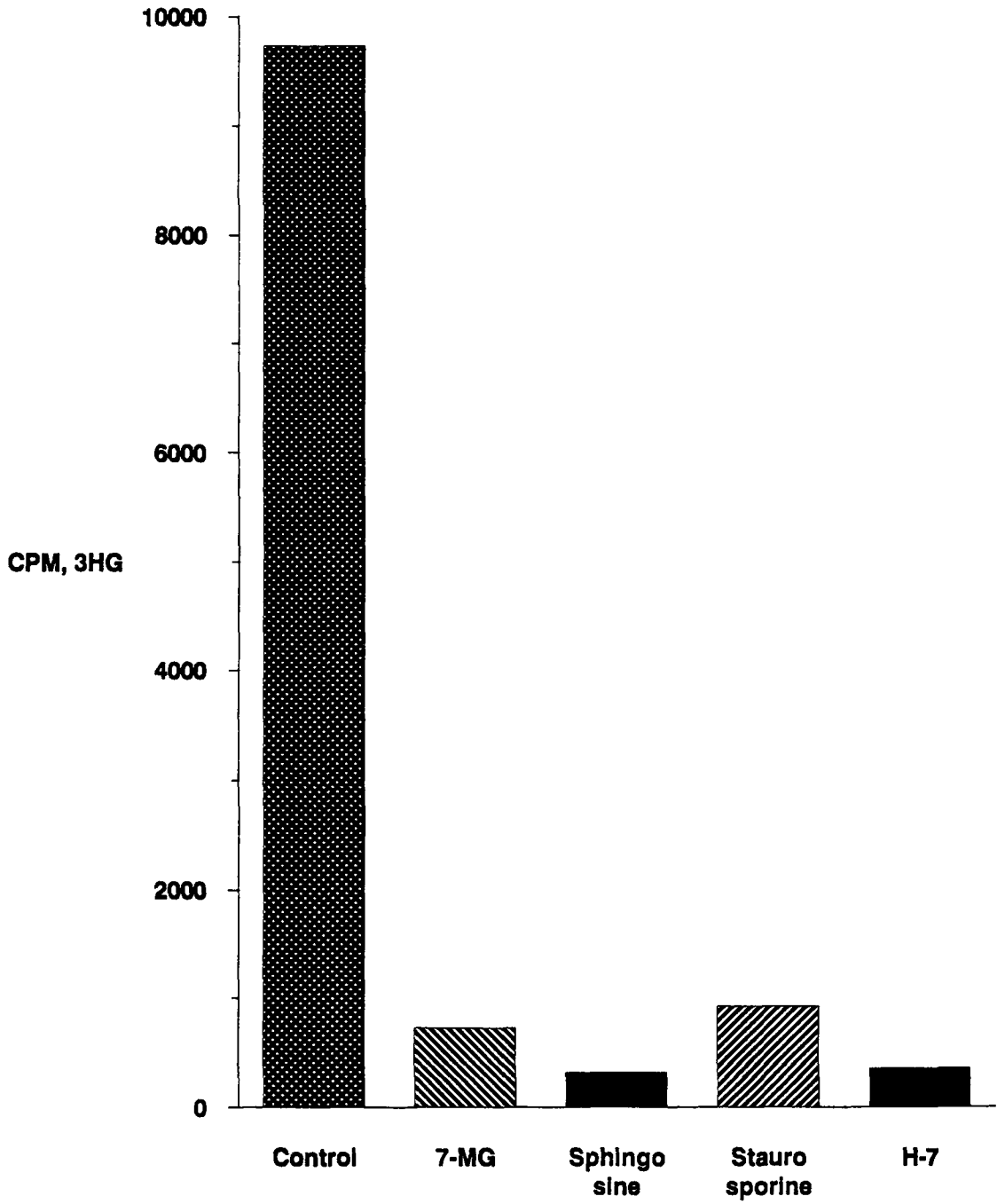
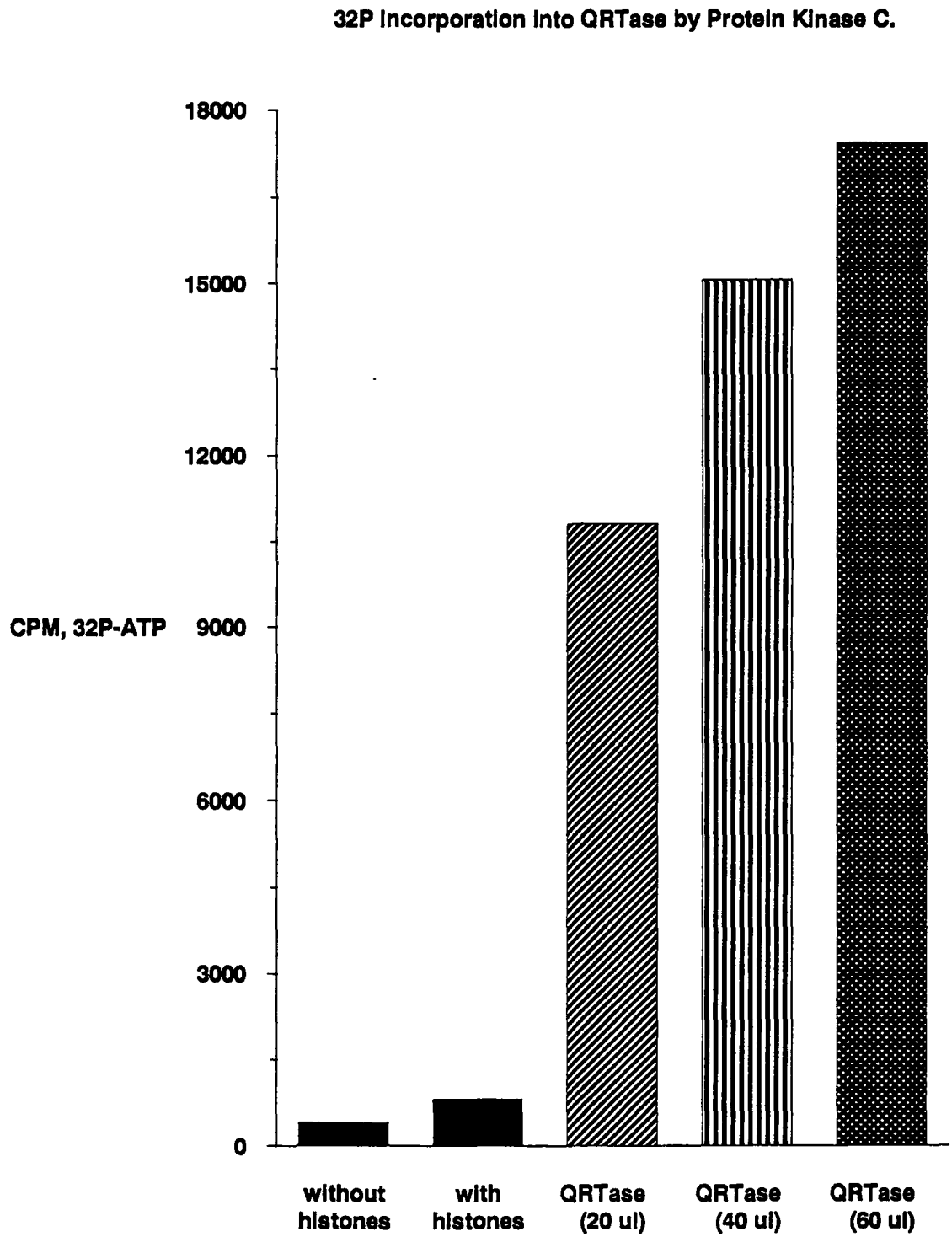


Figure 15. ^{32}P–ATP incorporation into QRTase by protein kinase C. 20 μl, 40 μl and 60 μl precipitated reaction mixture was tested to determine whether we can tag the insertion enzyme with ^{32}P–ATP.


32P Incorporation into QRTase by Protein Kinase C.
CPM, 32P-ATP
18000
15000
12000
9000
6000
3000
0
without histones
with histones
QRTase (20 ul)
QRTase (40 ul)
QRTase (60 ul)

DISCUSSION

This project involved the *in vitro* study of the enzymes protein kinase C and tRNA–guanine ribosyltransferase. The isolation and purification of active enzymes was essential in order to examine the dependence of the insertion enzyme on protein kinase C. The instability of the insertion enzyme is well known. It appears to have a short activity half–life resulting in a complete loss of activity within eight days when stored at –70 °C. Total reactivation of QRTase was accomplished when protein kinase C was added to the reaction mixture. This suggests that the insertion enzyme is regulated by protein kinase C. Furthermore, evidence of incorporation of ^{32}P into the QRTase fraction by protein kinase C shows that activation by protein kinase C is probably due to phosphorylation of QRTase. The presence of phosphatase enzyme activity was demonstrated during isolation of the insertion enzyme. The possibility exists that the cause of inactivation of QRTase is due to contamination with phosphatase that removed the activating phosphate moiety. It is conceivable that excess phosphatase activity may result in hypomodified tRNA *in vivo*.

Incorporation of radioactive guanine to tRNAs from Morris hepatoma, SV40–transformed cells, and hepatoma tRNA from cancer patients (1) demonstrated that tumors contain considerable amounts of tRNA with guanosine in place of queuosine, while tRNAs isolated from normal tissues were modified with respect to queuosine. This suggests that the insertion enzyme is involved with neoplasia. The observation of increased tRNA synthesis with queuosine

hypomodified tRNA in tumor cells was explained as a result of a decrease in substrate, queuine. However, addition of exogenous queuine to Ehrlich ascites tumor cells did not result in an increase of modification in tRNA with respect to queuosine. We can now offer a possible explanation of the inability of the enzyme to incorporate exogenous queuine, since protein kinase C appears to regulate the insertion enzyme.

We are able to relate our results to previous ones *in vivo* (27), suggesting that the link of hypomodified tRNA and neoplasia is the relative activity of the phosphorylating enzyme PKC. Previously reported results which demonstrated chronic exposure of human fibroblasts with phorbol esters down regulates the protein kinase C activity, due to action of a specific protease (27), and reduces the efficiency of the queuine uptake mechanism, resulting in queuine hypomodified tRNA, support our results *in vitro*. Our observation that *in vitro* protein kinase C regulates the insertion enzyme explains the fact that short term exposure of human fibroblasts to phorbol esters activates the phosphorylating enzyme and results in queuosine modified tRNA. In contrast, chronic exposure results in degradation of PKC by a specific protease and therefore, results in hypomodified tRNA.

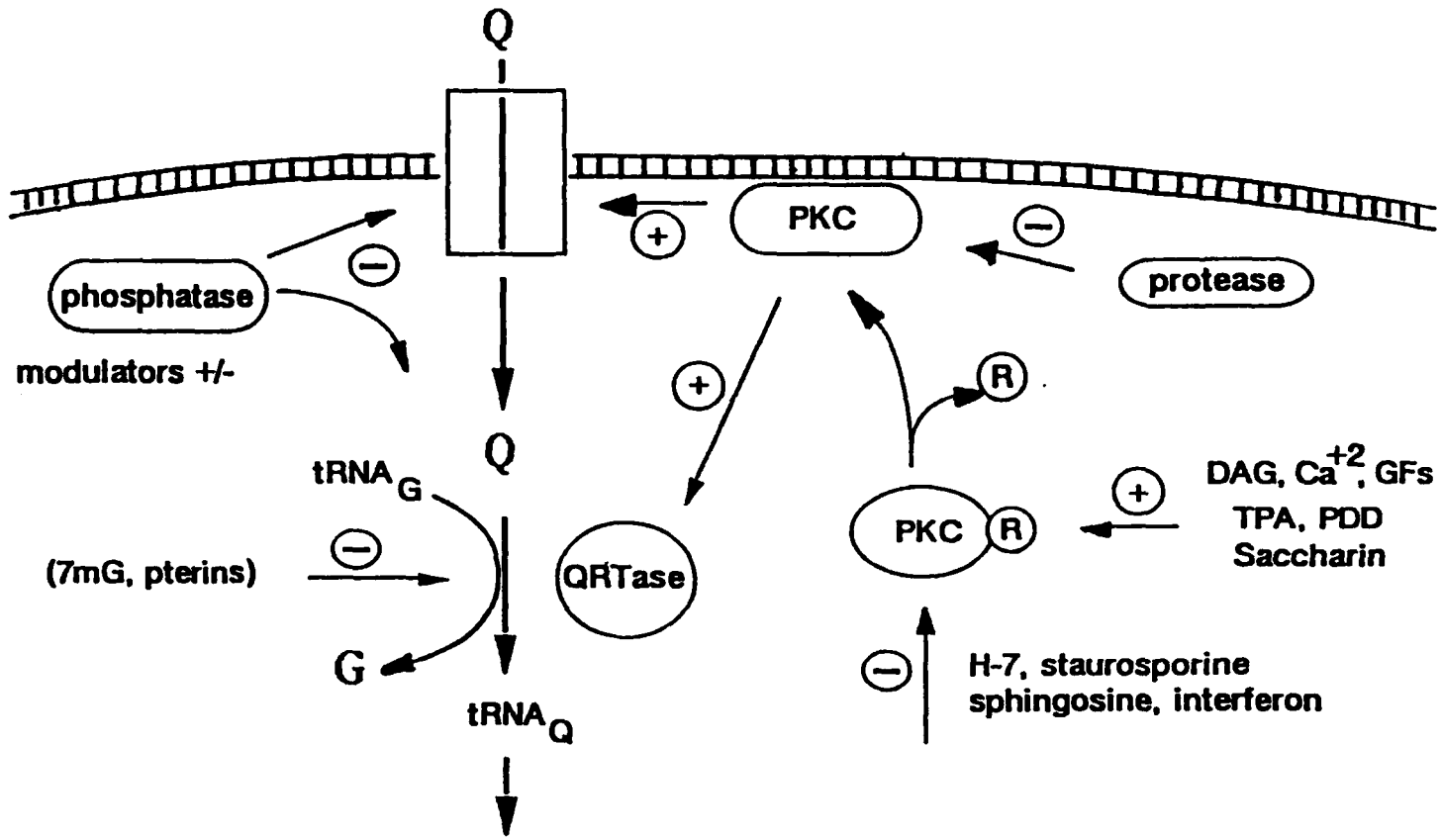
Further work could include the determination of the insertion enzyme's life time (half life) in storage, in the presence and absence of protein kinase C. Determination of the activity of the insertion enzyme in the presence of phosphatase with and without specific inhibitors against phosphatases would actually indicate that phosphatases are able to deactivate QRTase. This is another possible explanation for the presence of hypomodified tRNA in tumor cells. It is unknown, though, what actually happens to the insertion enzyme in tumor cells. Transfer RNA–guanine ribosyltransferase could be present and inactive, or its absence could indicate a gene control phenomenon. Further studies could be made to raise

antibodies against the protein and use fluorescence microscopy to reveal the insertion enzyme amount and location in the cell. Tumor cells may not be able to show binding of the antibodies to the cells, indicating the possibility of the absence of ribosyltransferase.

Figure 16 is a summary of established and hypothesized processes concerning the insertion enzyme. As shown, H–7, staurosporine, sphingosine and interferon are able to deactivate protein kinase C. Diacylglycerol, calcium, phorbol esters and saccharin activate protein PKC by inducing a proteolytic cleavage of its regulatory domain. The presence of a specific protease is able to degrade the phosphorylating enzyme as a result of chronic exposure to phorbol esters or other natural stimulating agents. An active protein kinase C will phosphorylate and therefore activate the queuine transport enzyme and the insertion enzyme. The presence of phosphatase enzymes was obvious during the QRTase isolation, indicating potential inactivation and another possible control point. Known inhibitors of QRTase, 7–methylguanine and pterins, will deactivate the enzyme resulting in hypomodified tRNA.

We propose that there is a sophisticated control over tRNA–guanine ribosyltransferase involving both a kinase, phosphatase mechanism and direct inhibition by 7–methylguanine and pterins. The insertion enzyme is responsible for the modification of tRNA and we have shown that it is activated *in vitro* by protein kinase C. Its loss of activity leads to hypomodified tRNA that is known to be present in tumor cells, suggesting that protein kinase C controls tRNA–guanine ribosyltransferase and therefore, controls tRNA modification.

Figure 16. Proposed model of the relationship between the phosphorylation enzyme and the insertion enzyme. Q–uptake and Q–modification is dependent on protein kinase C.


Q
PKC
phosphatase
protease
modulators +/-
R
Q
tRNA G
DAG, Ca+2, GFs
TPA, PDD
Saccharin
(7mG, pterins)
QRTase
PKC
R
G
H-7, staurosporine
sphingosine, interferon
tRNA Q

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