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INHIBITION OF N^8-ACETYLSPERMIDINE DEACETYLATION ACTIVITY

OF RAT LIVER

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University of Connecticut, 1978

A Thesis

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at

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This thesis, written and submitted by

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_____

Dated _____July 18, 1980_____

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INHIBITION OF N^8-ACETYLSPERMIDINE DEACETYLATION ACTIVITY OF

RAT LIVER

Abstract of Thesis

An enzyme activity located in the soluble fraction of rat liver which is known to deacetylate N^8-acetylspermidine is studied. Using a crude cytosol preparation from rat liver, tritium-labelled N^8-acetyl-spermidine $[\text{acetyl-}^3\text{H}]$ was shown to undergo deacetylation which was dependent upon both time of incubation and protein concentration. The Michalis constant for this deacetylation of N^8-acetylspermidine is approximately 4.4 μM.

N^1-Acetylspermidine showed a competitive inhibition of this N^8-acetylspermidine deacetylation activity, and was found to be the most potent inhibitor tested. Diamine and polyamine compounds were also shown to inhibit the deacetylation of N^8-acetylspermidine. Spermidine was the most potent inhibitor of the naturally occurring polyamines tested, followed in order by spermine, putrescine, and cadaverine. Of numerous acetylated compounds studied, only acetylprocainamide showed any inhibition of this N^8-acetylspermidine deacetylating activity at concentrations below 10.0 mM.

Possible functions of the deacetylating activity are discussed along with speculation of the role this enzyme activity may play _in vivo._

INTRODUCTION

Polyamines

Antony van Leeuwenhoek first discovered spermine phosphate crystals as components of human semen in 1678 (1). Within the next 100 years, similar polyamine substances were detected in other body fluids and in tissues. In 1926 Dudly, Rosenheim, and Rosenheim (2) established the structure of spermine, and one year later these workers reported the presence of spermidine in animal tissues (3). Interest in these so-called polyamines declined until the 1950's, when Rosenthal and Tabor (4) studied the pharmacology of both spermidine and spermine. Since that time, research in the area of polyamines has expanded immensely.

The term polyamine today refers not only to spermidine and spermine, but also to the diamine putrescine. Other diamines such as 1,3-diaminopropane and cadaverine (1,5-diaminopentane) are sometimes also included in this category of polyamines. Putrescine, spermidine, and spermine have been found in almost every living organism studied, including animals, plants, protozoa, and viruses (1). Distribution studies of polyamines within humans have shown that these compounds occur in most tissues studied, and they appear in highest concentration in the brain, adrenals, kidney, and liver (5). Human blood and urine have also been shown to contain polyamines (6,7). In animals,

as in humans, these polyamines have been found in virtually all tissues studied (1,8,9).

The observations cited above led to the natural question of the origin of these polyamines in the organisms studied. The biosynthetic pathway which has been elucidated shows that putrescine is a product of the decarboxylation of L-ornithine by the enzyme ornithine decarboxylase (10). Spermidine is then formed by the enzyme spermidine synthase, which transfers a propylamino group from decarboxylated s-adenosylmethionine and links it to putrescine. A similar reaction can be catalyzed by spermine synthase using spermidine as the propylamino acceptor in place of putrescine, and spermine is the resultant product (11). Each enzyme mentioned above appears to be located in the cytoplasmic fraction of the cell. The biosynthetic pathway discussed above was first demonstrated in the rat prostate gland, and has since been shown to occur in the developing chick embryo and the regenerating rat liver (12,13). Details of this pathway are shown in Figure 1.

Despite numerous studies, the physiological role of polyamines remains uncertain. Studies in vitro have shown that polyamines can stabilize the nucleic acids DNA and RNA (14), and this is demonstrated by the protection of these nucleic acids from thermal denaturation and enzymatic degredation (15,16). Polyamines have also been shown to stimulate RNA synthesis in intact rat liver nuclei (17). It has been postulated that the stabilizing effect which polyamines have on RNA may be an explanation of this observation. The stimulation of RNA synthesis was later reported in two in vivo systems: the developing chick embryo and the regenerating rat liver (18,19). The observations discussed above strongly suggest that polyamines lie in close association with nucleic acids in vivo. However, there is little direct evidence

Arginine

urea

L-Ornithine

CO_2 (1)

Putrescine

$(NH_2CH_2CH_2CH_2CH_2NH_2)$

+

S-Adenosylmethionine

CO_2

Decarboxylated
Adenosylmethionine

Methylthio-
adenosine,
H^+ (2)

Spermidine

$(NH_2CH_2CH_2CH_2NHCH_2CH_2CH_2CH_2NH_2)$

Methylthio-
adenosine,
H^+ (3)

Spermine

$(NH_2CH_2CH_2CH_2NHCH_2CH_2CH_2CH_2NH_2CH_2CH_2CH_2NH_2)$

Key: (1) ornithine
decarboxylase
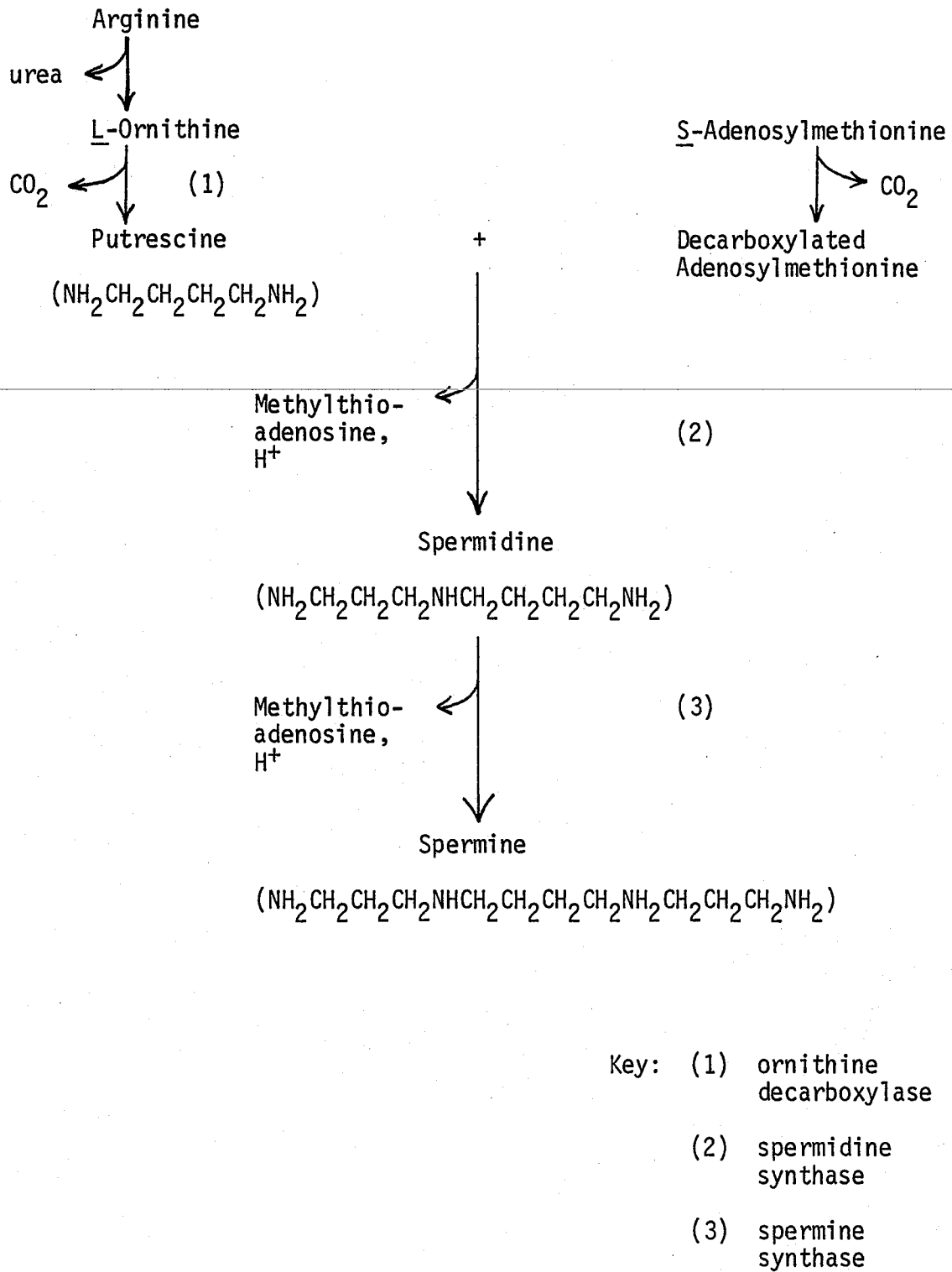
(2) spermidine
synthase

(3) spermine
synthase

FIGURE 1. BIOSYNTHETIC PATHWAY OF POLYAMINES

for this close association with the exception of experiments similar
to the study by Ames and Dubin (20). This experiment used a coliphage
to demonstrate that both putrescine and spermidine were indeed bound
to the phage DNA.

Recent work with polyamines has been focused on two areas: (i)
their occurrence and physiologic function in growing tissues, and (ii)
their presence and metabolism in the central nervous system (CNS).

Polyamines have been shown to be essential for the growth of cer-
tain viruses and bacteria (21,22). In animal tissues, most of the at-
tention has been focused on ornithine decarboxylase activity. Rapidly
proliferating cells have been shown to have high levels of ornithine
decarboxylase activity (23). This high level of ornithine decarbox-
ylase activity seems to result in production of increased levels of
putrescine and spermidine in tissues. Among those tissues which were
used to demonstrate increases in ornithine decarboxylase activity and
subsequent elevated polyamine levels were regenerating rat liver, rat
uterus after estradiol administration, and tissue which had undergone
malignant transformation (i.e., breast tumors) (23). The fact that
neoplastic tissues contain high levels of polyamines led Russell (24)
to attempt to show that elevated urinary output of polyamines in pa-
tients could serve as a diagnostic indicator for cancer. However,
recent reports indicate that urinary levels of polyamines are highly
variable, and not a reliable marker for detecting cancer (11).

Polyamine research in the central nervous system stems from the
findings of relatively high polyamine concentrations in the brain tis-
sue of a variety of animals. Within the CNS, spermidine is found
associated mainly with the brain stem and spinal cord, whereas spermine
is much more uniformly distributed (25). The idea of spermidine and

spermine acting as neurotransmitters or neuromodulators is a popular one, and this idea is certainly supported by the fact that these polyamines are found in the synaptosomal fraction of in vitro brain cell preparations (26). However, further research is necessary before any conclusions can be drawn as to the functions of these polyamines in the CNS.

Polyamine metabolism studies in the CNS show that spermine becomes deaminated in chicken brain, possibly by spermine oxidase (27). Kakimoto (28) discovered the new polyamine putreanine [N-(4-aminobutyl)-3-aminopropionic acid] in mammalian CNS, thought to be another metabolite of polyamines. Seiler (29) has demonstrated using brains of trout and mice that putrescine can become metabolized to ɣ-aminobutyric acid (GABA). This same study further demonstrated that putrescine may also become acetylated. Seiler and Al-Therib (30) showed that this monacetylputrescine can undergo oxidative deamination both in vitro and in vivo by monoamine oxidase (MAO) to form N-acetyl-ɣ-aminobutyric acid and eventually GABA via a deacetylation reaction.

Acetylated Polyamines

Research on polyamine metabolism outside the CNS has primarily centered around the oxidation of these amines. Diamine oxidase has been reported to deaminate putrescine (11). Non-specific amine oxidases from plasma and semen have been shown to metabolize polyamines (1). Furthermore, Holtta (31) has recently shown that a specific polyamine oxidase can be found in rat liver.

In contrast to the above studies, the acetylation of polyamines has received relatively little attention. Polyamines were shown to become acetylated in Esherichia coli by Dubin and Rosenthal (32).

Perry (33) reported that acetylputrescine was found in human brain tissue, and Seiler (34) later revealed that putrescine can be acetylated in vitro by rat brain as well as kidney and liver preparations. Moreover, this acetylation was found to be associated with the nuclei of the liver cell. Acetylspermidine was reported by Walle (35) to be present in the urine of cancer patients. More recently, acetylputrescine, acetylcadaverine, and two naturally occuring acetylated spermidines (N^1- and N^8-acetylspermidines) were found to be present in the urine of normal patients (36). N^1-Acetylspermidine has also been shown to occur in normal human serum (37).

Blankenship studied the in vitro acetylation of spermidine and spermine using chromatin from rat liver and kidney cells (38). This acetylation activity was found to be due to an acetyltransferase which required acetyl-CoA for the donation of the acetate group. Libby (39) studied two acetyltransferases from calf liver nuclei which were able to acetylate both spermidine and the histones. There has been disagreement as to which group of compounds, the polyamines or the histones, are the natural substrates for these acetylating enzymes (Blankenship, personal communication).

While studying the in vitro acetylation of polyamines, Blankenship (38) noticed that the addition of the 100,000g supernatent fraction from rat liver caused the disappearance of both N^1-and N^8-acetylspermidine. Libby (40) found this disappearance to be due to a deacetylating enzyme which occurs in rat liver cytosol. This enzyme was partially purified and was found to deacetylate N^1-and N^8-acetylspermidine along with acetylspermine, but not acetylputrescine. In addition, all three polyamines inhibited this deacetylation competitively. Blankenship (41) showed that this deacetylation activity was the highest in the liver

but was also present in spleen, kidney, and lung. Using a crude cytosol preparation, N^8-acetylspermidine was found to be deacetylated much more readily than N^1-acetylspermidine. This lack of N^1-acetyl-spermidine deacetylation was possibly due to an alternate route of metabolism, perhaps that of polyamine oxidase, which is known to metabolize N^1-acetylspermidine (42). In order to fully understand the deacetylation of polyamines, a brief review of the known deacetylating enzymes is necessary.

Deacetylating Enzymes

Acetylation of drugs occurs primarily in the soluble fraction of the liver by acetyltransferases (43). Acetyl-CoA has been found to donate the acetate group, and drugs which are acetylated are almost exclusively the aromatic amines (44). Deacetylation, on the other hand, is a term which can be taken to include an enormous number of reactions. For instance, an acetylcholinesterase certainly does deacetylate acetylcholine. Also, it is known that aspirin is deacetyl-ated by numerous esterases, some of which are located in the liver (45). However, the esterase group of enzymes has been exhaustively studied (46) and will not be dealt with in this report. Concern here is for the hydrolysis of the amide bond to form the corresponding free amine and acetic acid.

Deacetylation studies on aliphatic amines have been primarily confined to acetylated amino acids. Michel (47) showed that rat liver and kidney were capable of deacetylating various amino acids, such as N-acetylglycine and N-acetyl-DL-leucine. Bray (48) demon-strated that this amino acid deacetylating activity was different than the deacetylation activity associated with the metabolism of

acetanilid. Recently, Endo (49) has demonstrated that there is a specific enzyme located in rat kidney which deacetylates only acetylated aromatic amino acids, but not a aliphatic amino acids.

Acetanilid has been the prototype drug used in deacetylation studies of acetylated aromatic compounds (47,48). Michel (47) reported the deacetylation of acetanilid by liver homogenates, and Kirsch (50) demonstrated that acetanilid deacetylase activity was located in hog liver microsomes. Nimmo-Smith used chick-kidney mitochondria to demonstrate the deacetylation of acetanilid and over 20 derivatives of this molecule, suggesting that the deacetylase in the chick kidney was rather non-specific. However, Keise (52) performed an extensive study which used acetanilid to compare the deacetylation activities of different animals, different tissues, and different subcellular preparations. The conclusions from this study were that the liver seemed to be the organ with the highest acetanilid deacetylation activity, that this activity was found predominately in the soluble fraction of the liver cell, and that there is an enormous variation among species with respect to their ability to deacetylate acetanilid.

Other studies on compounds which become deacetylated are remarkably scarce. Reasons for this apparent lack of interest are that (i) the amide bond can usually undergo hydrolysis in an acid media, which allows for hydrolysis by the stomach when these compounds are ingested, and (ii) the fact that many of the early researchers showed that these amidohydrolases were quite non-specific (53). However, there have been other deacetylation studies conducted which deserve mention here. Phenacetin was reported by Brodie and Axelrod (54) to undergo deacetylation in human liver. Although only a minor fraction of the entire amount of phenacetin is deacetylated by the liver, the product formed by

deacetylation has been shown to be involved in the oxidation of hemoglobin to methemoglobin. This phenacetin deacetylation activity is located in the microsomal fraction of liver cell homogenates as reported by Ber hammer (55). Kampffmeyer (56) further demonstrated that pretreating dogs with phenobarbital caused an induction of these microsomal deacetylating enzymes.

Walasezek (57) showed that colchicine becomes deacetylated by mouse and rat liver. Acetylaminofluorine, a known carcinogen, has been reported to be deacetylated by rat liver slices (58). Krebs (59) has shown that sulfonamide drugs are acetylated and subsequently deacetylated by sheep liver and kidney. Unfortunately, there has been no follow-up research which would show whether these deacetylating activities represent the same enzyme activity or if the enzymes responsible are distinct and specific for their particular substrate.

Today it is generally agreed that there are many different N-deacetylating enzymes, most of which are found in the soluble fraction of liver cells, and some of which have broad substrate specificities The purpose of the present study is to determine the specificity of the observed N^8-acetylspermidine deacetylating activity, and to report an apparent Michalis constant, or K_m, for N^8-acetylspermidine. Acetylated compounds which are known to undergo deacetylation will be used to determine if they may cause inhibition of this N^8-acetylspermidine deacetylation activity. Finally, parent polyamine compounds will be used to examine the possibility that they may also cause an inhibition of this deacetylation activity.

MATERIALS AND METHODS

Chemicals and reagents used were purchased from J.T. Baker Chemical Company, Phillipsburg, New Jersey. Drugs were obtained commercially from Sigma Chemical Company, St. Louis, Missouri. Tritium-labeled acetic anhydride (5.0 mCi, Lot #1147-080, 10.2 mg, activity 47.3 mCi/mmole) was purchased through New England Nuclear, Boston, Massachusetts.

Synthesis of N^8-Acetylspermidine [acetyl-^{3}H]

^{3}H-Acetic anhydride (1×10^{-4} moles) was reacted with a tenfold excess of spermidine in 1 ml of acetonitrile. The reaction was carried out for 1 hour in a dry ice bath. After the reaction was complete, 10 ml of distilled water was added and this mixture was applied to the column described below for separation.

Purification of N^8-Acetylspermidine [acetyl-^{3}H]

A 25 cm x 1.5 cm column packed with Amberlite ion exchange resin CG-50 (100-200 mesh) was prepared with a pH of 9.5 by washing with 3N NH_4OH and distilled water. This column was then used to separate un-reacted spermidine, N^1- and N^8-monoacetylated spermidines, and other acetylated spermidine products, as described by Blankenship (38). After adding 10 ml of the reacted mixture to the column, elution was carried out with 50 ml distilled water followed by 100 ml of 0.5M NH_4OH and 100 ml of 2.0M NH_4OH. Fractions containing 2.0 ml were

collected during the 2.0M NH_4OH elution, and each odd fraction was analyzed by thin-layer chromatography. The fractions were then checked for radioactive content by liquid scintillation spectrometry. Fractions found to contain only the N^8-acetylspermidine were pooled and the NH_4OH was removed by evaporation. The viscous solution remaining after evaporation was taken up in 10 ml distilled water and the pH was adjusted to 2.0 with 1N HCl. This product was found to remain stable when kept refrigerated.

Purity Determination of N^8-Acetylspermidine [acetyl-^{3}H]

Purity of the final N^8-acetylspermidine dihydrochloride was checked using thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). For TLC, 5 ul of the final product was spotted onto a 20 cm x 5 cm silica gel plate (type 60F-254, 0.25 mm thickness, Brinkman) along with 5 μl of standard N^8-acetylspermidine. Chromatography was carried out with chloroform-methanol-NH_4OH (2:2:1), and polyamines were identified with ninhydrin (20 mg/ml ninhydrin in butanol). The silica gel was then scraped for the area corresponding to the R_f value of N^8-acetylspermidine, and this was placed into a scintillation vial. The remainder of the material on the plate was then scraped into a separate scintillation vial, and both vials counted for radioactivity. Purity was estimated by the ratio of counts of N^8-acetylspermidine/ total counts on the entire plate.

Methods of sample preparation for HPLC are described under "Quantitation of N^8-acetylspermidine by HPLC." N^8-Acetylspermidine was checked for contamination by N^1-acetylspermidine by collection of 0.8-ml fractions with retention times corresponding to that of the N^1-acetylspermidine peak. These 0.8-ml fractions were placed in separate scintillation vials and counted. Fractions (0.8 ml) were also collected corresponding

to the N^8-acetylspermidine peak and these were placed in separate scintillation vials and counted. Contamination was estimated from the ratio of total counts corresponding to the N^1-acetylspermidine peak/ total counts in all fractions collected.

Cytosol Preparation

Naive male Wistar rats (Simonsen Labs, Gilroy, California), 140-180 grams, were stunned and decapitated. The liver was removed and suspended in cold 0.25M sucrose-50mM NaH_2PO_4-5mM $MgCl_2$ buffer (SPM buffer), pH 7.4. Three grams of liver were then placed in 12 ml of SPM buffer and homogenized for 15 seconds at the maximum setting of a Brinkman Polytron (model PCU-2) homogenizer. The homogenate was centrifuged in a Beckman Ultracentrifuge (model L3-40) at 105,000 x g for 60 minutes. The resulting supernatant was diluted 1:1 with SPM buffer and used in the deacetylation assay. All cytosol samples were prepared fresh at 4^0 C.

Enzyme Assays

The procedure for assaying N^8-acetylspermidine deacetylation was carried out by the methods of Blankenship (41). A final volume of 0.5 ml consisted of 0.125 mmoles of sucrose, 29 μmoles of NaH_2PO_4, 0.3-0.7 nmoles of N^8-acetylspermidine, and 0.5-1.3 mg protein (from liver cytosol preparation). The reaction was initiated by the addition of 1:1 cytosol-SPM mixture. This was incubated at 37^0 C for 10 minutes while shaking using a Dubnoff incubator. The reaction was stopped at 4^0 C by the addition of 100 μl 1N HCl-0.05M acetic acid. For the inhibition studies, inhibiting substances were added in SPM buffer in concentrations ranging from 0.10mM to 10.0mM.

Extraction Procedure

Ethyl acetate (3.0 ml) was added to each of the tubes containing
the completed reaction mixture. The tubes were then shaken vigorously
for 1 hour, and centrifuged at 1500 $\underline{x}$ g for 5 minutes. Two ml of the
organic layer was transferred to a counting vial containing 10 ml of
scintillation fluid, and radioactivity was determined by liquid scintil-
lation spectrometry using a Beckman CPM 100 scintillation counter. The
scintillation fluid was toluene: Triton X 100 (2:1) which contained 3.66
grams/liter of 2,5-diphenoxazole and 0.66 grams/liter of $\underline{p}$-bis [2-
(5-phenoxazole)] -benzene. Counts per minute were converted to disintegra-
tions per minute using a quench-correction curve. The standard used for
the quench-correction contained [^{3}H] -toluene (Lot #697-199-3; activity 2.19
x 10^6 dpm/ml, calibrated 6/16/78), quenched with an acetone-chloroform
mixture (1:1) in increasing amounts.

Protein Determination

The amount of protein used in each assay was determined by the method
of Lowry (60). A standard curve was plotted using bovine serum albumin
(300 ug/ml) in 15 μg, 30 μg, and 60 μg amounts. The 1:1 cytosol-SPM mix-
ture from each assay was then diluted 1:9 with SPM buffer (to achieve
values within the range of the standard curve) and 50 μl of this dilution
was assayed at the same time as the standard curve was obtained. After 30
minutes absorption was measured on a double-beam spectrophotometer (model
ACTA C11) using a blank which lacked only protein. Measurements were
taken at λ of 750 mμ and a slit width value of 0.5.

Quantitation of N^8-Acetylspermidine by HPLC

Acetylated polyamines were prepared for HPLC analysis by the methods

described by Lettes (61). N^8-Acetylspermidine $[acetyl-^3H]$ and acetylated polyamine standards were diluted with perchloric acid giving a final concentration of 0.2N perchloric acid. Aliquots of 0.5 ml were saturated with sodium bicarbonate, three volumes of dansyl chloride in acetone were added (10 mg/ml, 56 μmoles), and this mixture was shaken overnight. Proline was added (to remove excess dansyl chloride), and the dansylated amines were extracted with toluene. The extract was evaporated to dryness, and the residue was resuspended in 50 μl of chloroform for HPLC analysis. Separation of dansylated derivatives was achieved on a Perkin Elmer Silica B/5 (0.46 x 25 cm) column with a Silica A (0.26 x 25 cm) precolumn. A Perkin Elmer Series 2 HPLC system utilizing a fluorescence detector (model 204-A) was used with a solvent of chloroform: isopropanol (75:2) at a flow rate of 0.4 ml/min.

Quantitation was established by structuring a standard curve of chromatograph peak areas vs. amount of standard N^8-acetylspermidine (0.5 nmole - 10.0 nmole range) present. Three 100 μl samples of synthesized N^8- acetylspermidine $[acetyl-^3H]$ were prepared as above and injected onto the column separately, the area of each peak determined, and the nmoles of N^8-acetylspermidine present calculated from the standard curve using the average peak area of the three trials.

RESULTS

Purification and Quantitation of N^8-Acetylspermidine [acetyl-^{3}H]

Radioactivity of the fractions which eluted off the column with 2.0 M NH_4OH is shown in Figure 2. Fractions 7-21 were pooled and 97.2% of total radioactivity had an R_f value corresponding to N^8-acetylspermidine on TLC. Contamination of this N^8-acetylspermidine by N^1-acetylspermidine was estimated by HPLC to be less than 2.6% of total radioactivity.

Pooled fractions containing N^8-acetylspermidine (3.6 x 10^5 dpm) were injected into the HPLC system, the peak area corresponded to approximately 6.9 nmoles of standard N^8-acetylspermidine, which represents a specific activity of 5.26 x 10^4 dpm/nmole.

Time and Protein Dependence of N^8-Acetylspermidine Deacetylation

N^8-Acetylspermidine deacetylation activity was shown to be linear for at least 15 minutes using 3.4 nmoles of N^8-acetylspermidine and 1.0 mg protein, as shown in Figure 3. This N^8-acetylspermidine deacetylation was found to be linear with respect to protein concentration up to 3.0 mg/assay as described in Figure 4, where 3.4 nmoles N^8-acetylspermidine were used with a 10-minute incubation time.

Determination of the K_m for Deacetylation of N^8-Acetylspermidine

Figure 5 represents the relationship between deacetylation velocity and N^8-acetylspermidine concentration (mM). The reaction is shown to

FIGURE 2.   SEPARATION OF N^1- AND N^8-ACETYLSPERMIDINE BY COLUMN CHROMATOGRAPHY

An elution profile is shown for N^1- and N^8-acetylspermidine $[$acetyl-^{3}H$]$ using a chromatographic system of Amberlite CG-50, eluted with 2 N NH_4OH. The first peak corresponded upon further analysis to N^8-acetylspermidine (o——o) while the second peak corresponded to N^1-acetylspermidine (■——■).
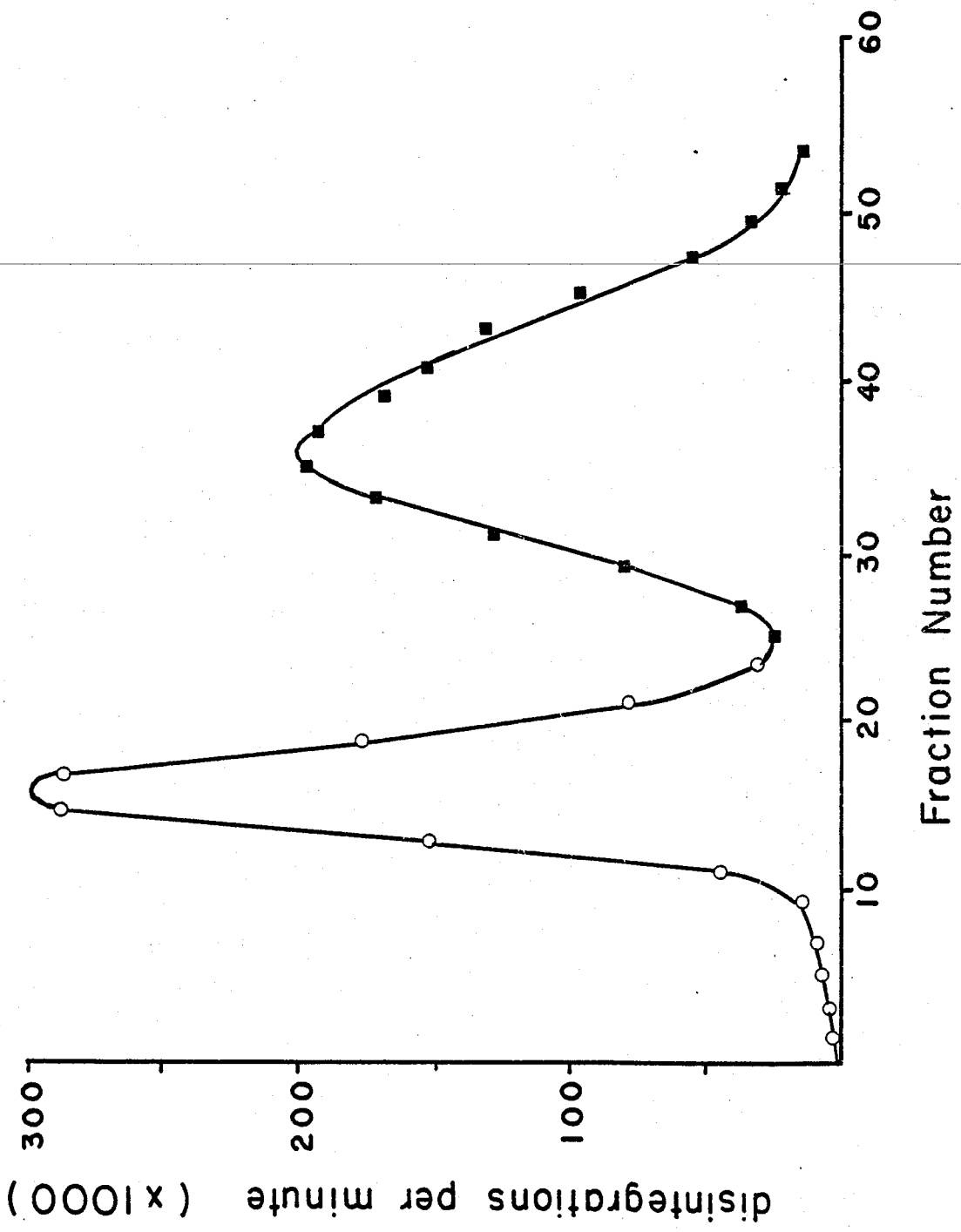
FIGURE 3.   TIME DEPENDENCE OF N^8-ACETYLSPERMIDINE DEACETYLATION

Experiments were run under standard incubation conditions at a concentration of 6.8 nmoles/ml of N^8-acetylspermidine $\left[\text{acetyl-}^3\text{H}\right]$.   Each point (■——■) represents the average of triplicate determinations.
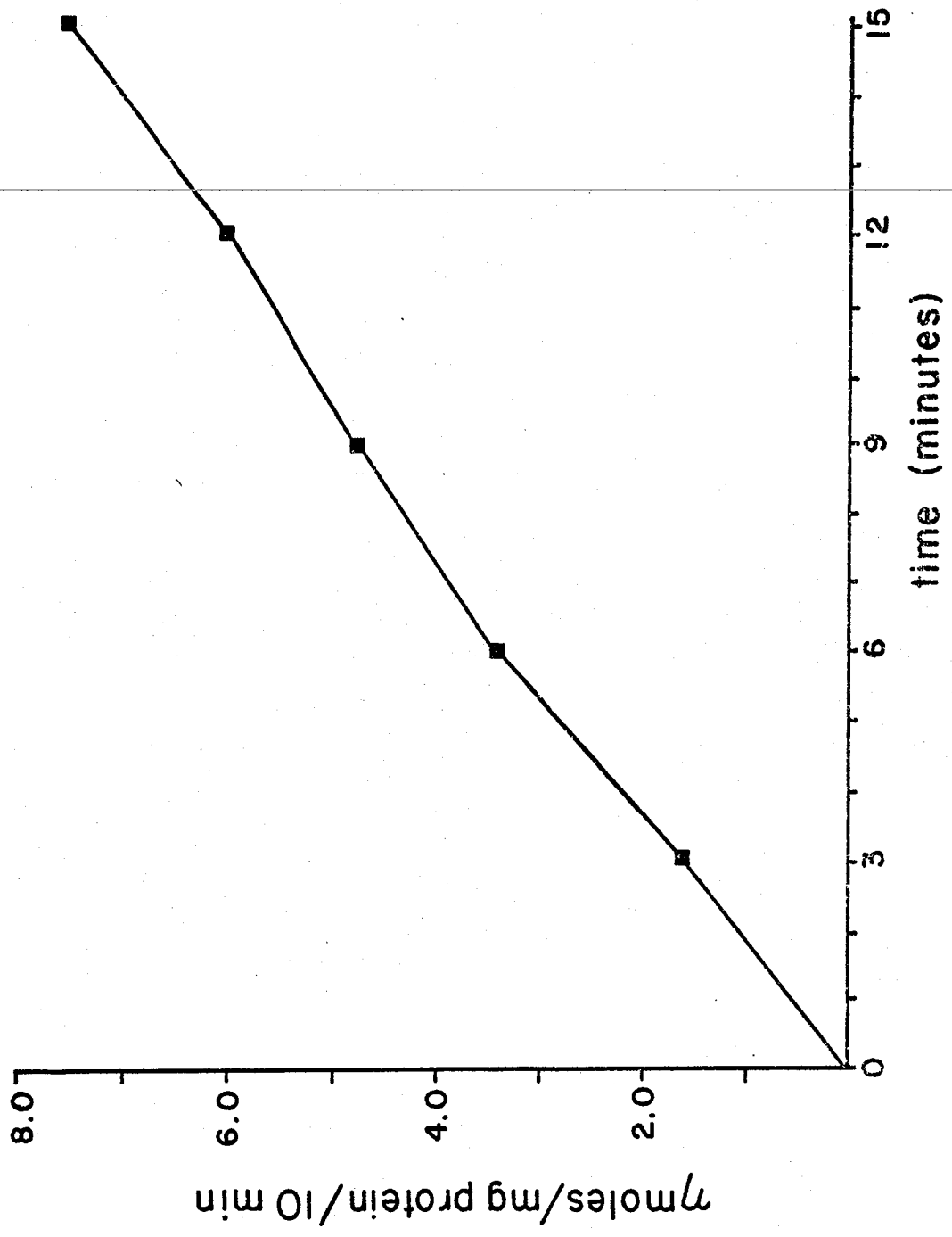
FIGURE 4. PROTEIN DEPENDENCE OF N^8-ACETYLSPERMIDINE DEACETYLATION

Experiments were run under standard incubation conditions at a concentration of 6.8 nmoles/ml of N^8-acetylspermidine $\left[\text{acetyl-}^3\text{H}\right]$. Each point (□——□) represents the average of triplicate determinations.
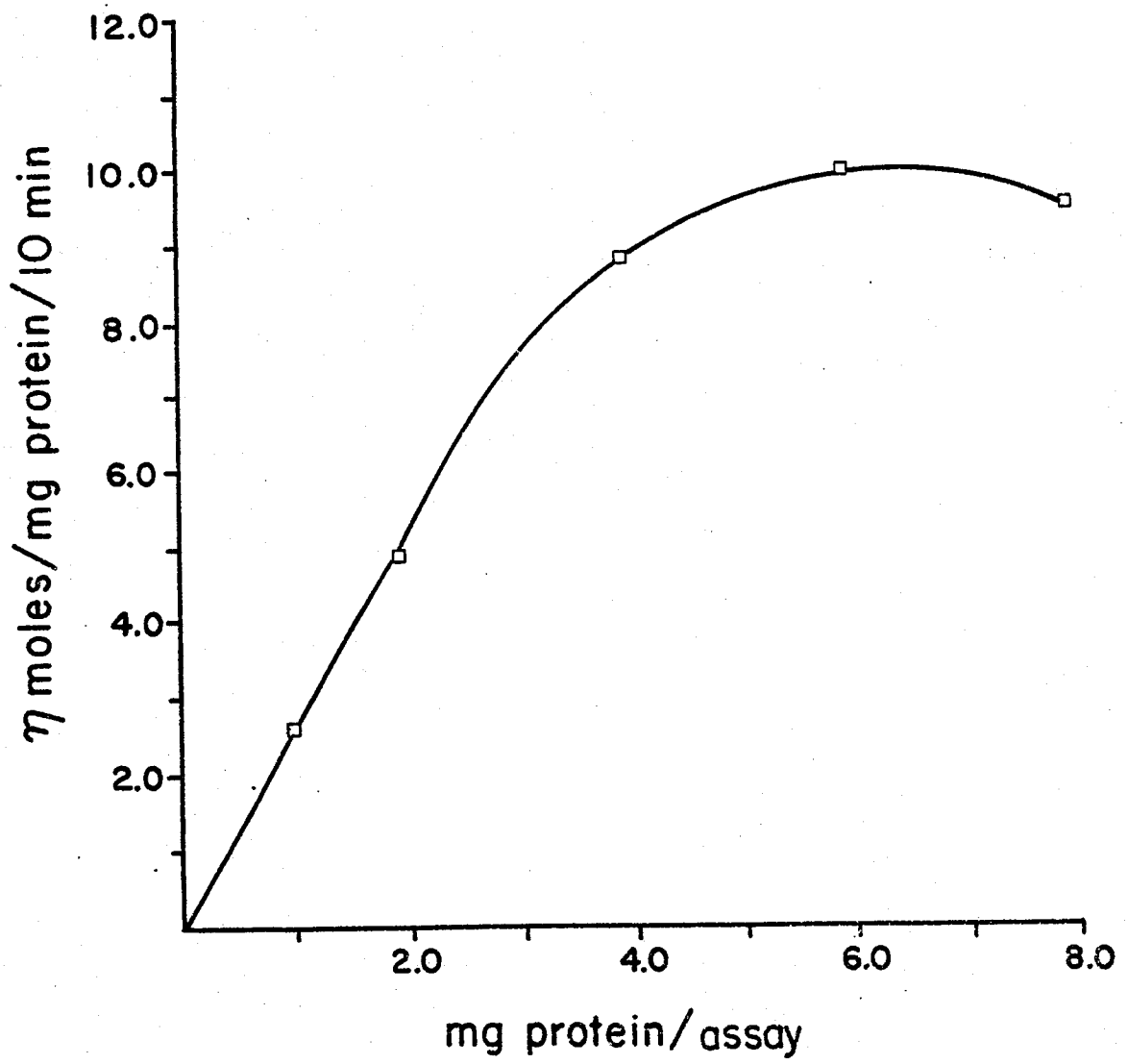
FIGURE 5. RELATIONSHIP OF N^8-ACETYLSPERMIDINE CONCENTRATION AND DEACETYLATION

ACTIVITY

Experiments were run under standard incubation conditions while varying the concentration of N^8-acetylspermidine $[\text{acetyl-}^3\text{H}]$. Each point (o—o) represents the mean of triplicate determinations.
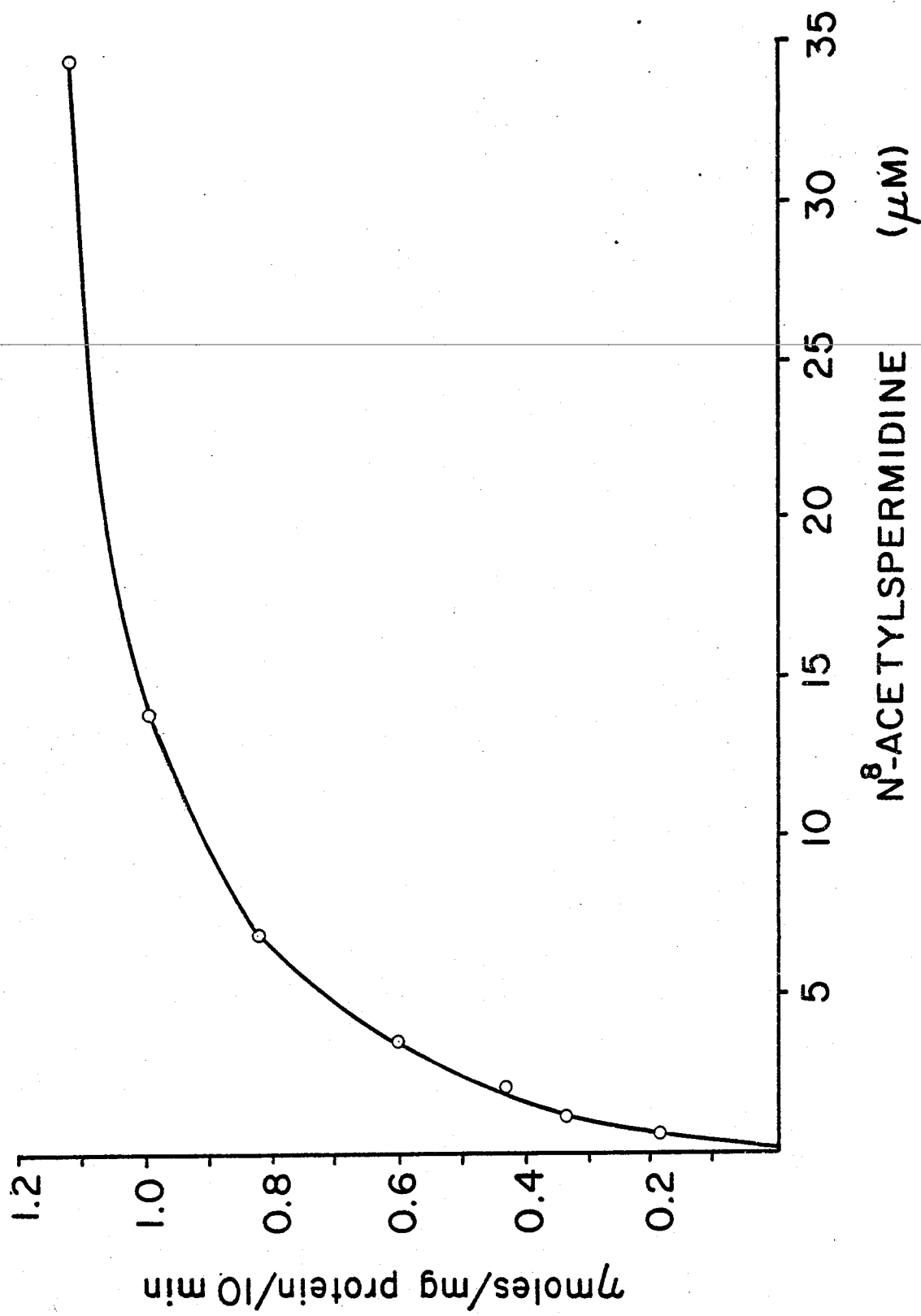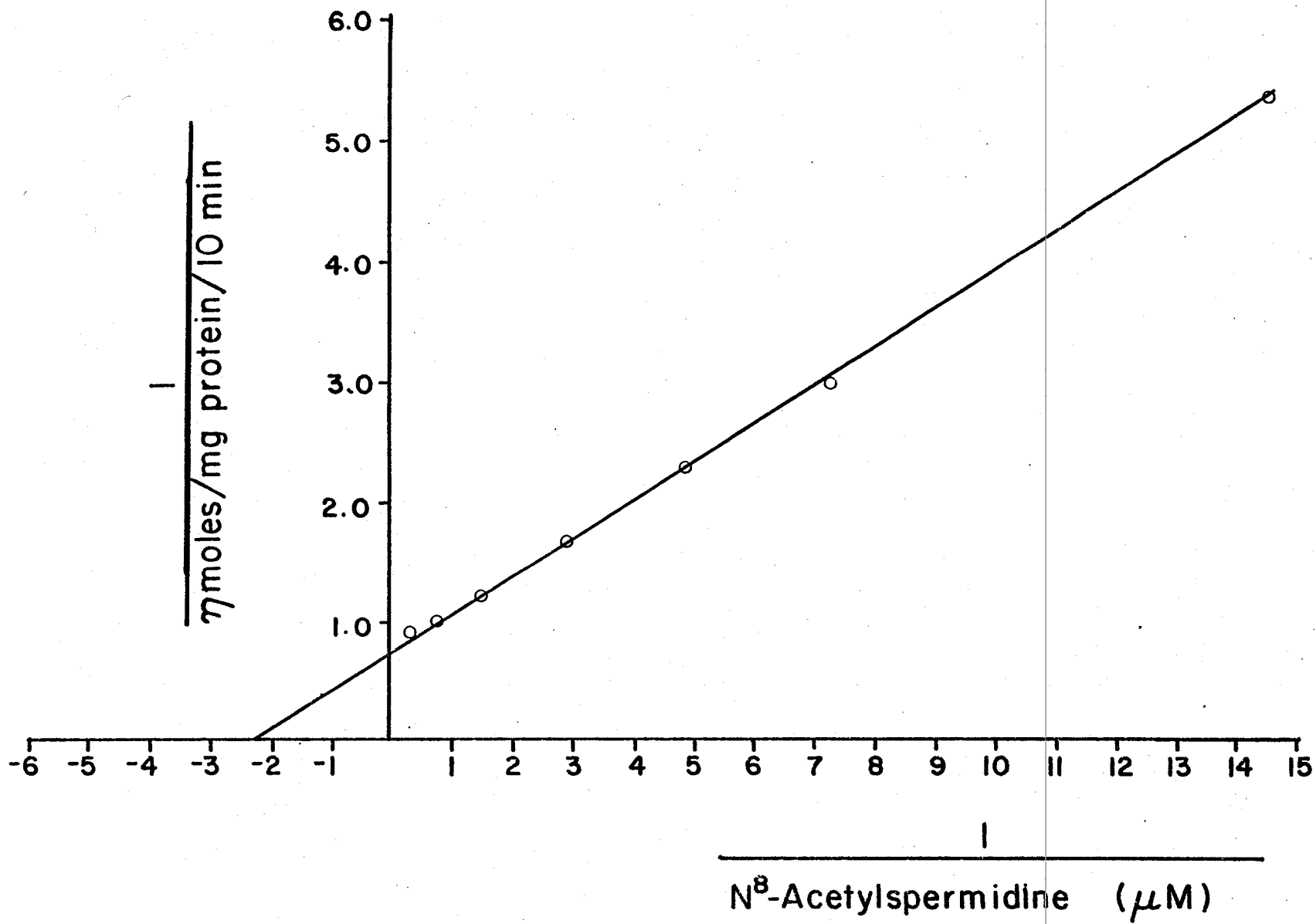
FIGURE 6. DOUBLE-RECIPROCAL PLOT OF N^8-ACETYLSPERMIDINE CONCENTRATION

VERSUS DEACETYLATION ACTIVITY

Experiments were run under standard incubation conditions at varying con-

centrations of N^8-acetylspermidine $[\text{acetyl-}^3\text{H}]$. Each point (o——o) repre-

sents the mean of triplicate determinations. This line has been fitted by

linear regression analysis (r=0.99).

proceed from a rate which follows first-order kinetics (which represent substrate concentration as the limiting factor) to a rate which follows mixed first-order and zero-order kinetics, and finally proceeding to a rate which follows zero-order kinetics (which represent enzyme concentration as the rate-limiting factor). Figure 6 shows a Lineweaver-Burk plot from which the value of K_m for N^8-acetylspermidine is estimated to be 4.4 μM. To insure that deacetylation of N^8-acetylspermidine was being measured, non-radioactive N^8-acetylspermidine standard was added to this reaction and was shown to inhibit this deacetylation competitively, with a K_i value of 4.5 μM.

Inhibition of N^8-Acetylspermidine Deacetylation by Various Compounds

Double-reciprocal plots were used to show inhibition of the N^8-acetylspermidine deacetylation by certain compounds (Figures 7-14). These graphs represent only the compounds which inhibited deacetylation when used at concentrations less than 10 mM. N^1-Acetylspermidine showed the most inhibition of any compound tested, and was the only substance which appeared to produce a purely competitive inhibition, as seen in Figure 7. Of the non-acetylated diamines and polyamines tested, spermidine showed the greatest ability to inhibit the deacetylation reaction (Figure 8). Spermine, putrescine, cadaverine, and 1,6-diamino-hexane all showed inhibition to a lesser extent as seen in Figures 9-12. Interestingly, 1,3-diaminopropane showed only slight inhibition at concentrations less than 10 mM. Acetylprocainamide (Figure 13) and aspirin (Figure 14) were the only drugs tested which caused inhibition of the deacetylating reaction at concentration less than 10 mM.

Acetanilid and sodium salicylate showed no inhibition at any of the concentrations tested (0.5-10.0 mM), whereas colchicine, phenacetin,

FIGURE 7. DOUBLE-RECIPROCAL PLOT OF THE INHIBITION OF N^8-

ACETYLSPERMIDINE DEACETYLATION BY N^1-ACETYLSPERMIDINE

Experiments were run under standard incubation conditions. The
three plots represent activity in the absence of N^1-acetylspermidine
(o——o) and in the presence of 0.1 mM (△——△) or 1.0 mM (□——□)
N^1-acetylspermidine. Each point represents the average of triplicate
determinations. All lines were fitted by linear regression analysis
(respective r values=0.99, 0.99, and 0.99).

FIGURE 8.   DOUBLE-RECIPROCAL PLOT OF THE INHIBITION OF N^8-

ACETYLSPERMIDINE DEACETYLATION BY SPERMIDINE

Experiments were run under standard incubation conditions.   The

three plots represent activity in the absence of spermidine (o——o)

and in the presence of 0.1 mM (△——△) and 0.3 mM (□——□) spermidine.

Each point represents the average of triplicate determinations.   All

lines were fitted by linear regression analysis (respective r values=

0.99, 0.99, and 0.99).

FIGURE 9.   DOUBLE-RECIPROCAL PLOT OF THE INHIBITION OF N^8-

ACETYLSPERMIDINE DEACETYLATION BY SPERMINE

Experiments were run under standard incubation conditions. The

three plots represent activity in the absence of spermine (o——o)

and in the presence of 0.1 mM (△——△) or 0.5 mM (□——□) spermine.

Each point represents the average of triplicate determinations. All

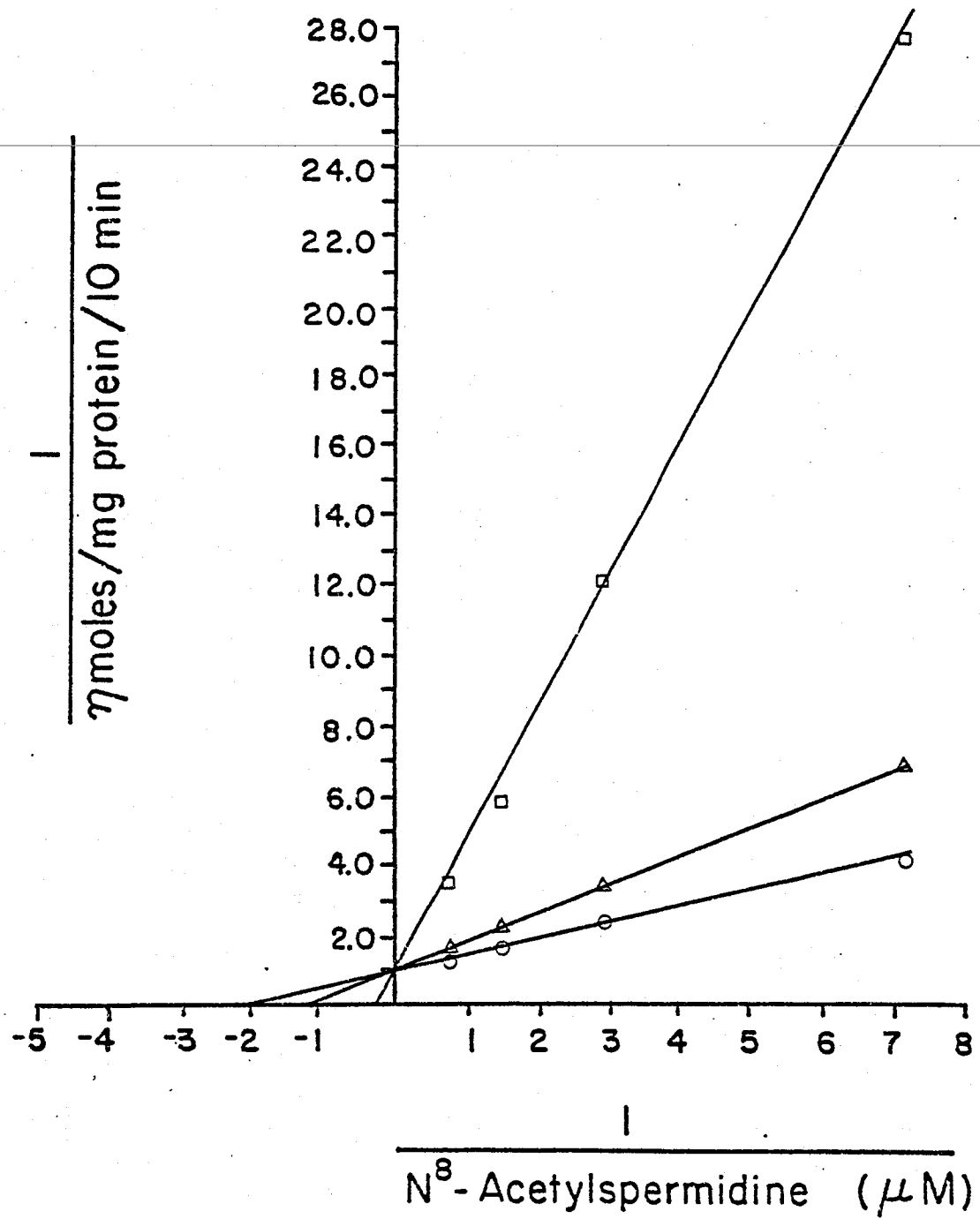lines were fitted by linear regression analysis (respective r values=

0.99, 0.99, 0.99).

FIGURE 10. DOUBLE-RECIPROCAL PLOT OF THE INHIBITION OF N^8-

ACETYLSPERMIDINE DEACETYLATION BY PUTRESCINE

Experiments were run under standard incubation conditions. The

three plots represent activity in the absence of putrescine (o——o)

and in the presence of 0.1 mM 9 (△——△) or 0.5 mM (□——□)

putrescine. Each point represents the average of triplicate

determinations. All lines were fitted by linear regression analysis
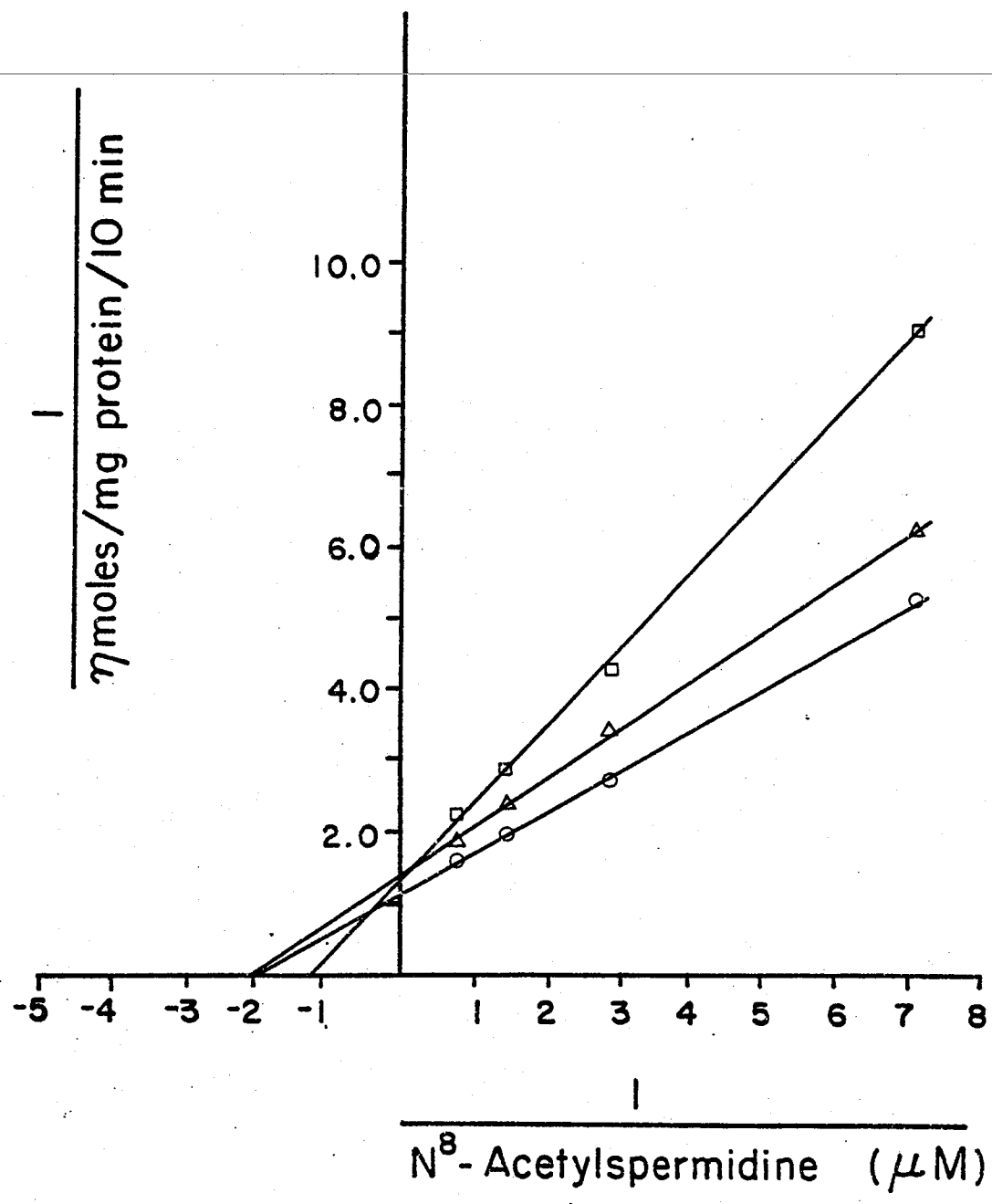
(respective r values=0.99, 0.99, and 0.99).

FIGURE 11.   DOUBLE-RECIPROCAL PLOT OF THE INHIBITION OF N^8-

ACETYLSPERMIDINE DEACETYLATION BY CADAVERINE

Experiments were run under standard incubation conditions. The

three plots represent activity in the absence of cadaverine (o——o)

and in the presence of 0.5 mM (△——△) or 1.0 mM (□——□) cadaverine.

Each point represents the average of triplicate determinations. All

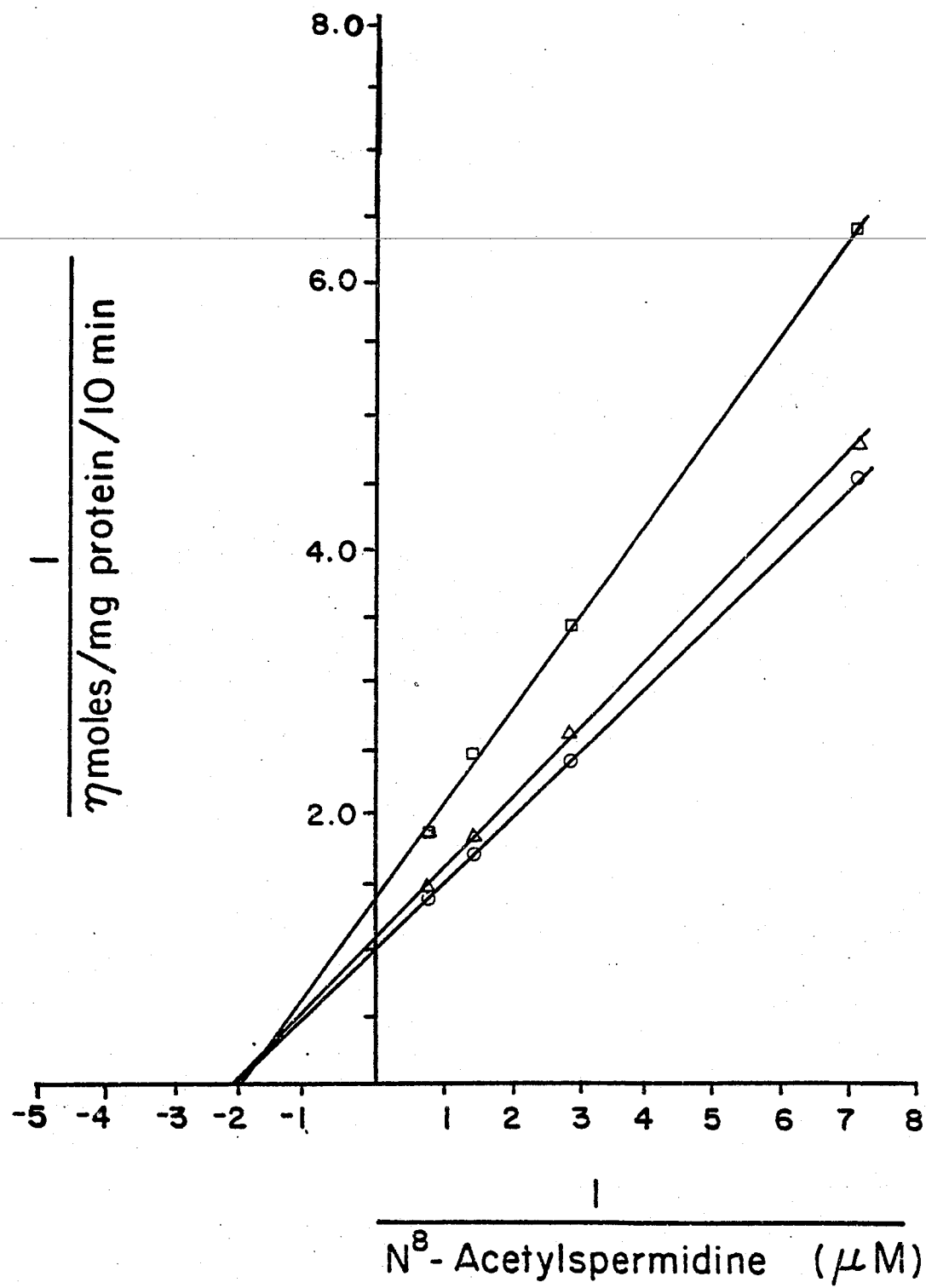lines were fitted by linear regression analysis (respective r values=

0.99, 0.99, and 0.99).

Figure: Double-reciprocal (Lineweaver-Burk) plot with y-axis labeled $\dfrac{1}{\eta\text{moles/mg protein/10 min}}$ (values 1.0–7.0) and x-axis labeled $\dfrac{1}{N^8\text{-Acetylspermidine}}\ (\mu M)$ (values −5 to 8).

FIGURE 12. DOUBLE-RECIPROCAL PLOT OF THE INHIBITION OF N^8-

ACETYLSPERMIDINE DEACETYLATION BY 1,6-DIAMINOHEXANE

Experiments were run under standard incubation conditions. The three plots represent activity in the absence of 1,6-diaminohexane (o——o) and in the presence of 0.5 mM (△——△) or 1.0 mM (□——□) diaminohexane. Each point represents the average of triplicate determinations. All lines were fitted by linear regression analysis (respective r values= 0.99, 0.99, and 0.99).
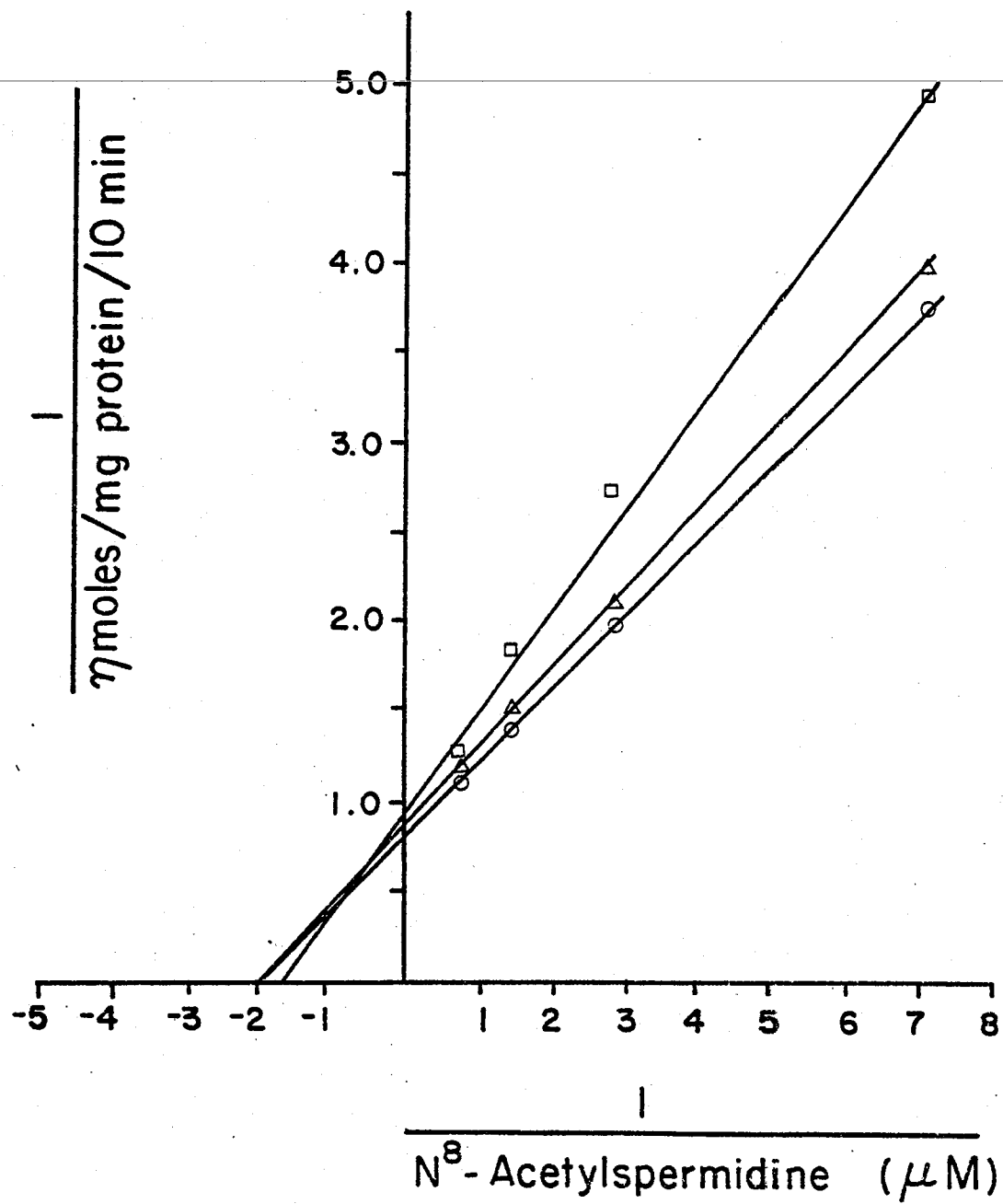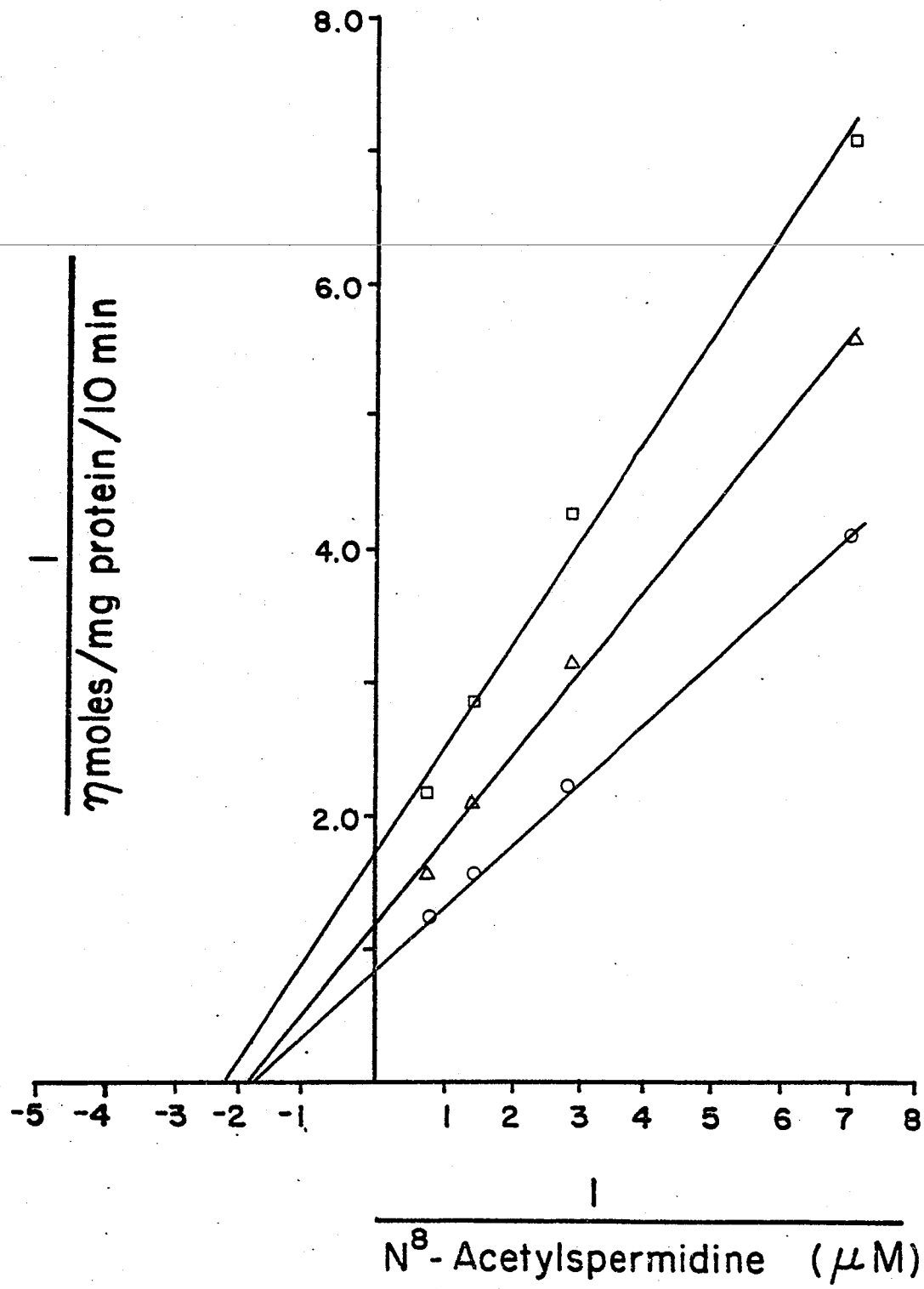
FIGURE 13. DOUBLE-RECIPROCAL PLOT OF THE INHIBITION OF N^8-

ACETYLSPERMIDINE DEACETYLATION BY ACETYLPROCAINAMIDE

Experiments were run under standard incubation conditions. The three plots represent activity in the absence of acetylprocainamide (o——o) and in the presence of 1.0 mM (△——△) or 10.0 mM (□——□) acetylprocainamide. Each point represents the average of triplicate determinations. All lines were fitted by linear regression analysis (respective r values=0.99, 0.99, and 0.99).
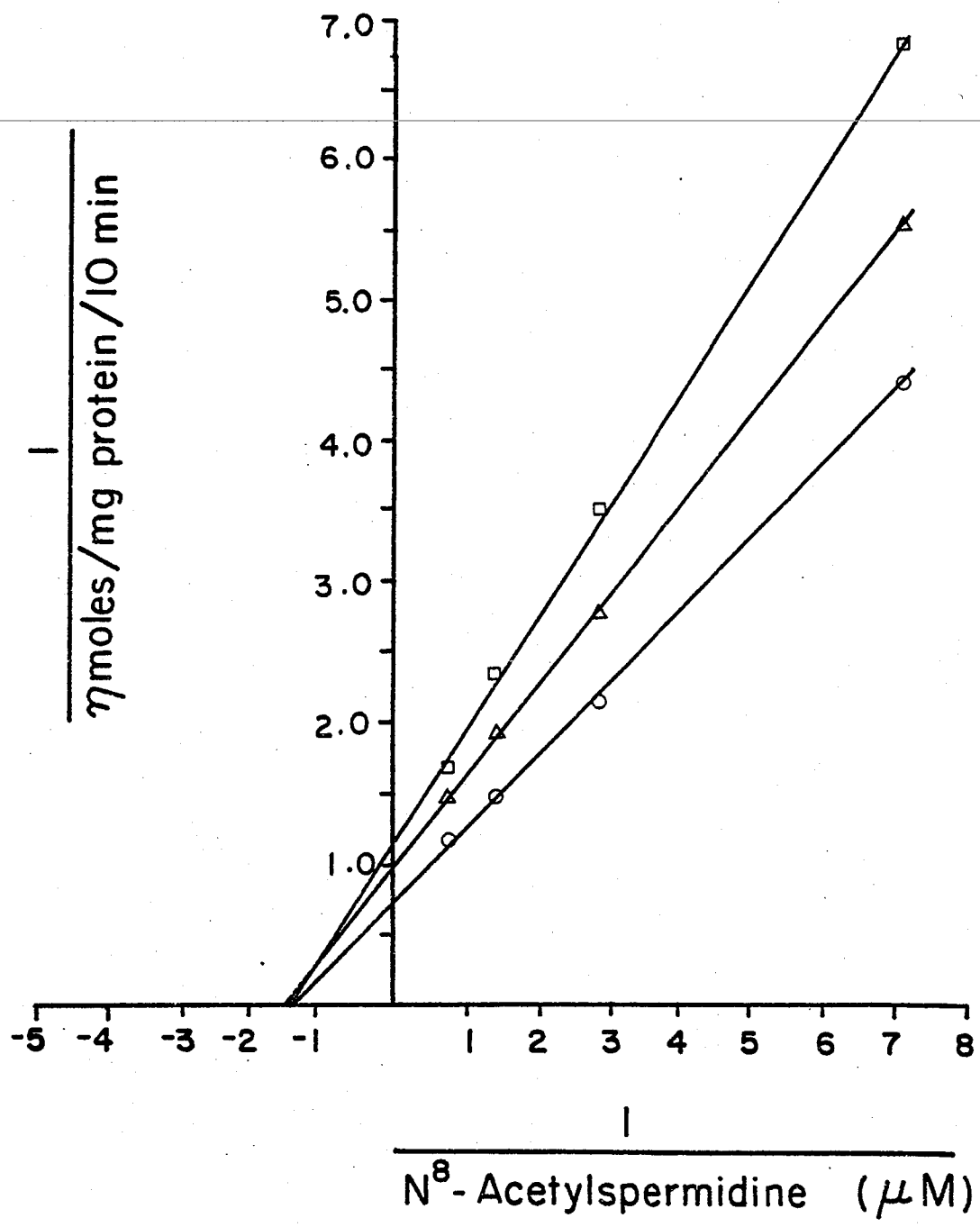
FIGURE 14. DOUBLE-RECIPROCAL PLOT OF THE INHIBITION OF N^8-

ACETYLSPERMIDINE DEACETYLATION BY ASPIRIN

Experiments were run under standard incubation conditions. The

three plots represent activity in the absence of aspirin (o——o)

and in the presence of 1.0 mM (△——△) or 10.0 mM (□——□) aspirin.

Each point represents the average of triplicate determinations.

All lines were fitted by linear regression analysis (respective
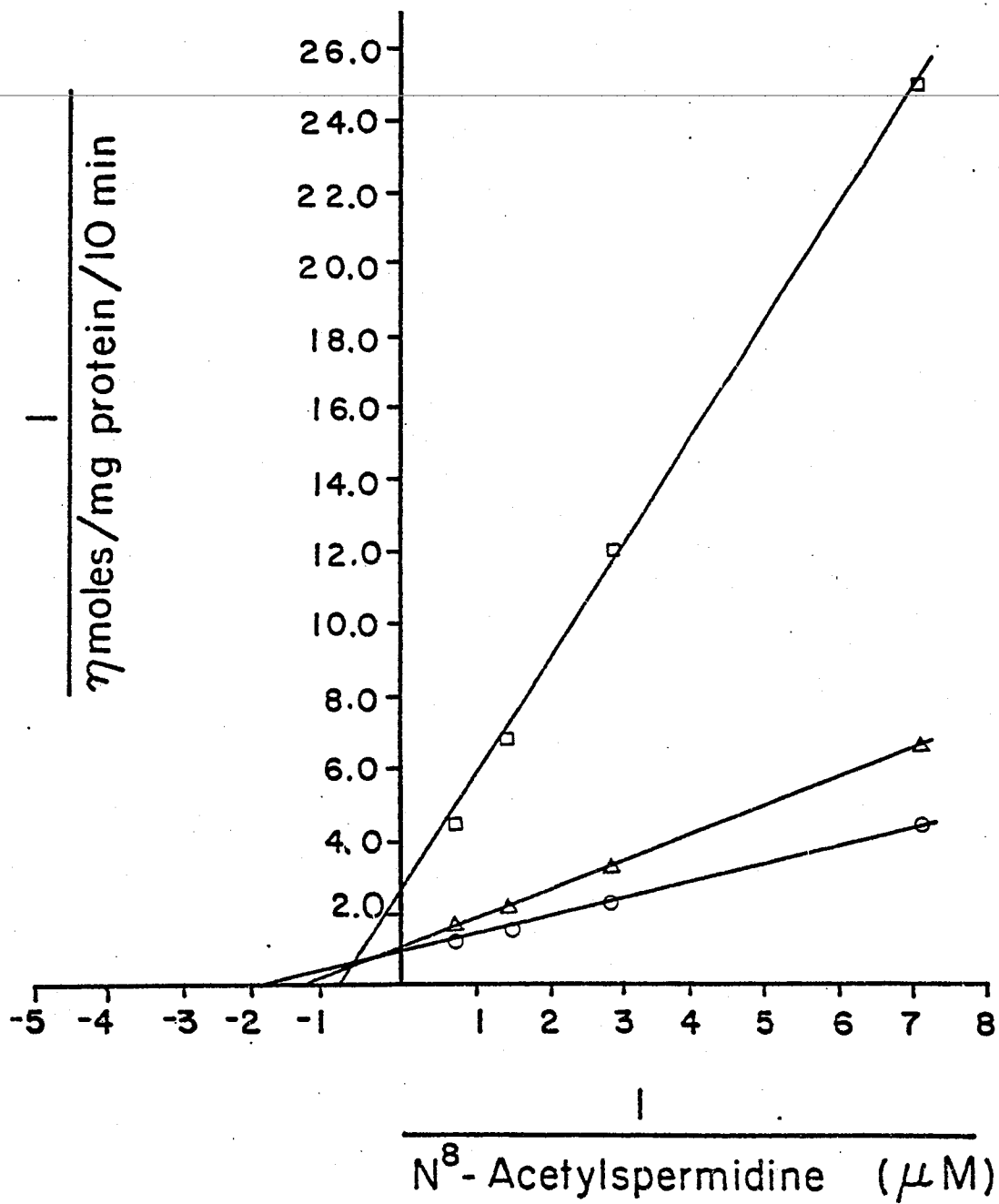
r values=0.99, 0.99, and 0.99).
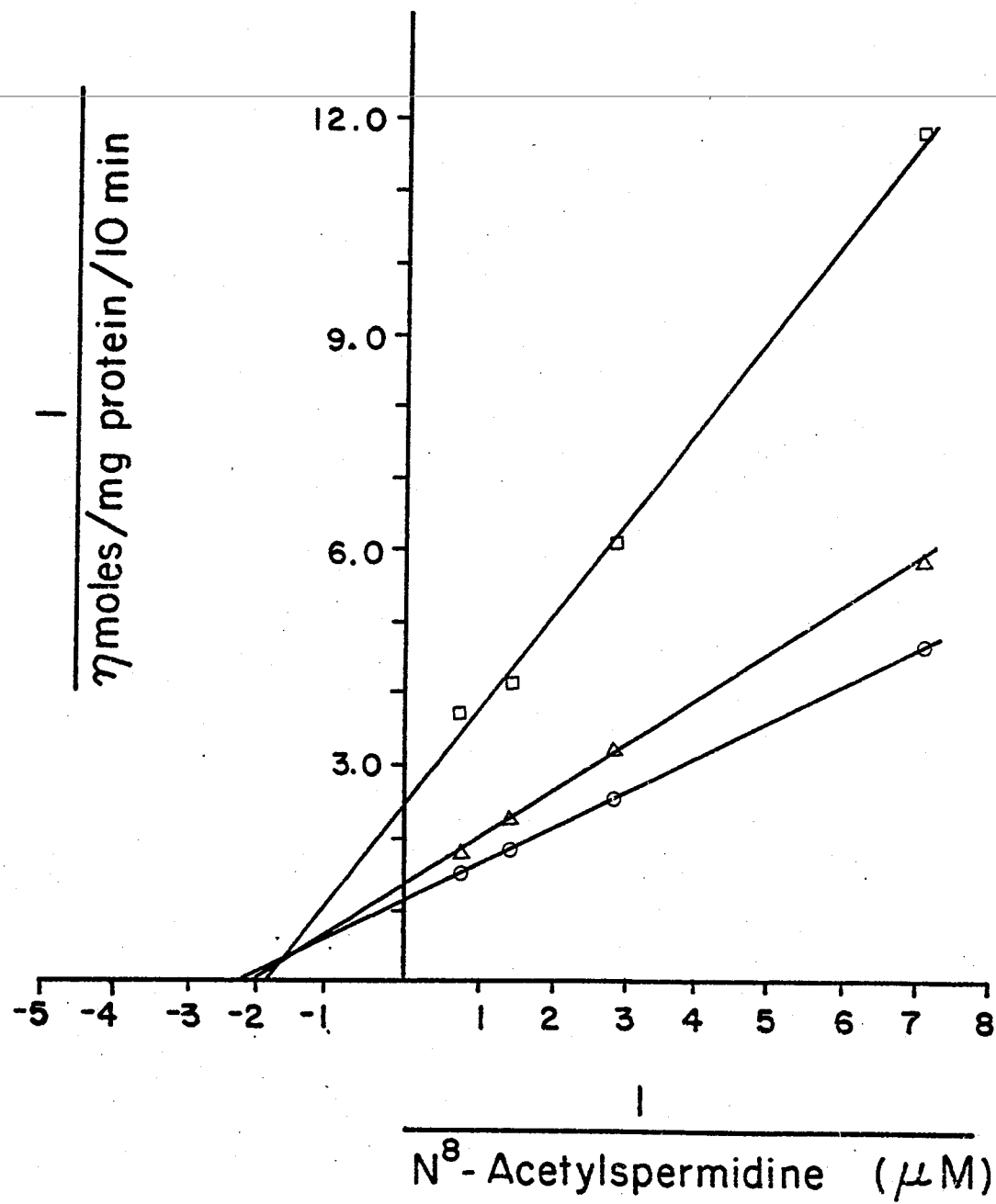
Table I. Inhibition of N^8-Acetylspermidine Deacetylation by Various Compounds[a]

COMPOUND	CONCENTRATIONS TESTED, mM		K_i, mM
N^1-Acetylspermidine	0.1,	1.0	0.16
Spermidine	0.1,	0.3	0.31
Spermine	0.1,	0.5	1.15
Putrescine	0.1,	0.5	1.45
Cadaverine	0.5,	1.0	1.50
1,6-Diaminohexane	0.5,	1.0	1.50
Acetylprocainamide	1.0,	10.0	1.90
Aspirin	1.0,	10.0	>2.00

[a]Compounds are listed in the order of decreasing inhibition of N^8-acetylspermidine deacetylation activity. The value for K_i is obtained from the Dixon plot, which is a graph of the concentration of inhibitor vs the reciprocal of the velocity of deacetylation. The intersection of the lines which represent different concentrations of N^8-acetylspermidine corresponds to the negative K_i of that particular inhibitor for this deacetylation reaction.

FIGURE 15.   DIXON PLOT OF THE EFFECT OF N^1-ACETYLSPERMIDINE ON

N^8-ACETYLSPERMIDINE DEACETYLATION ACTIVITY

Experiments were run under standard incubation conditions. The

three plots represent deacetylation activity using 0.69 nmoles ($\blacksquare$——$\blacksquare$),

1.72 nmoles ($\triangle$——$\triangle$), or 3.45 nmoles ($\square$——$\square$) of N^8-acetylspermidine

$\left[\text{acetyl-}^3\text{H}\right]$. Each point represents the average of triplicate

determinations. All lines were fitted by linear regression analysis

(respective r values=0.99, 0.99, and 0.99).

FIGURE 16. DIXON PLOT OF THE EFFECT OF ACETYLPROCAINAMIDE ON N^8-

ACETYLSPERMIDINE DEACETYLATION ACTIVITY

Experiments were .run under standard incubation conditions. The

three plots represent deacetylation activity using 0.69 nmoles (■——■),

1.72 nmoles (△——△), or 3.45 nmoles (□——□) of N^8-acetylspermidine

$\left[\text{acetyl-}^3\text{H}\right]$. Each point represents the average of triplicate

determinations. All lines were fitted by linear regression analysis

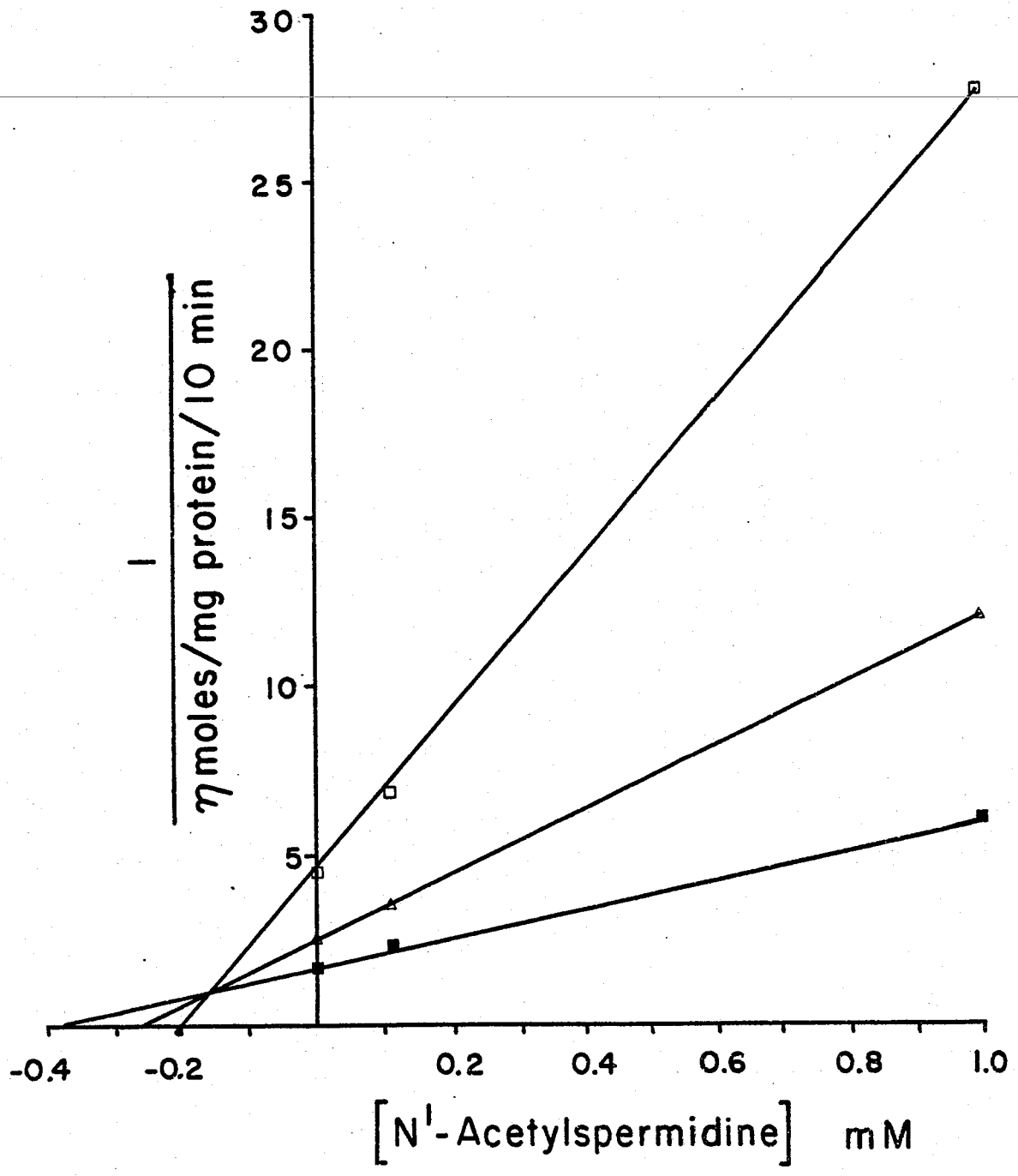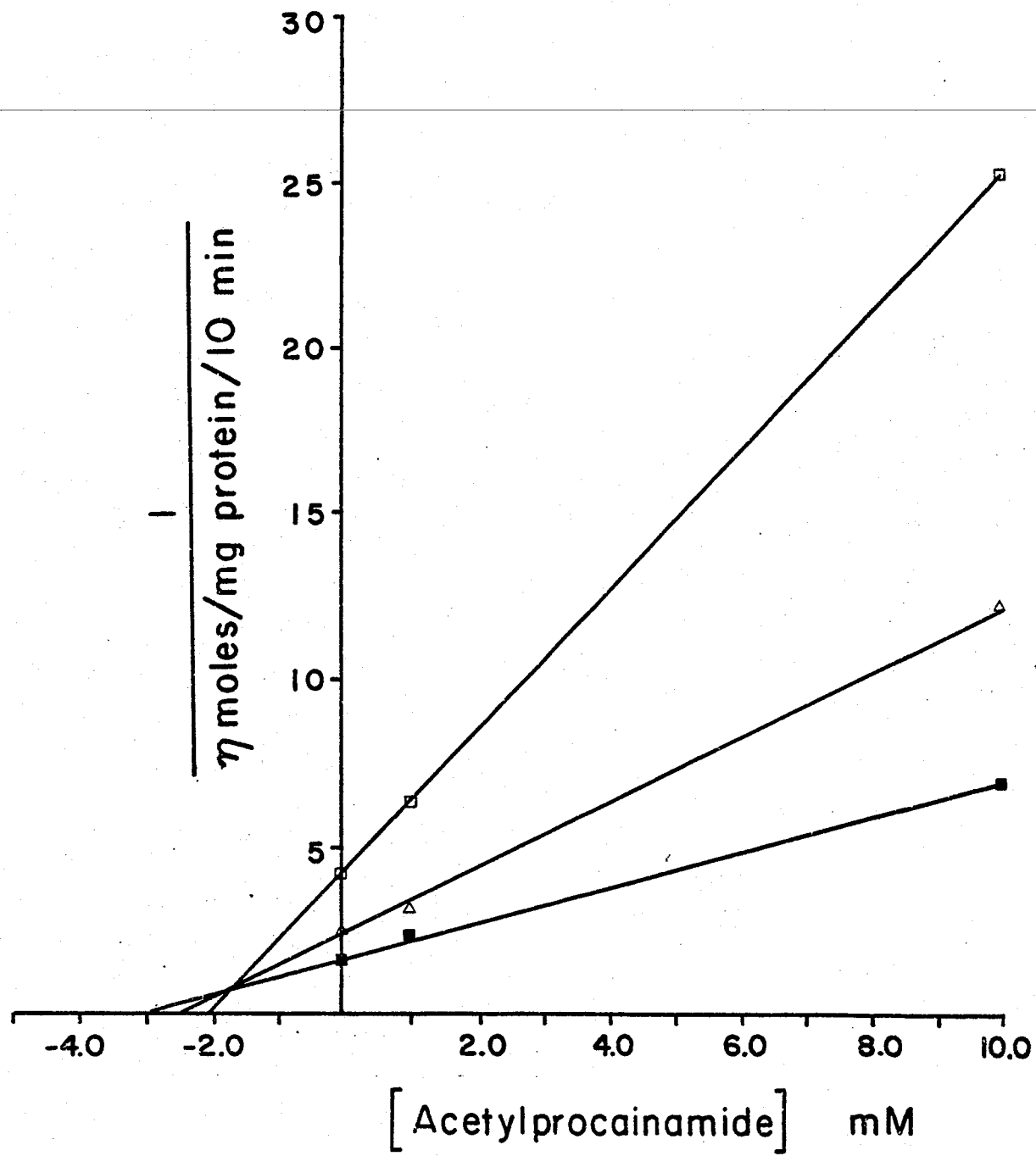(respective r values=0.99, 0.99, and 0.99).

neostigmine, acetylpenicillamine, and acetylacetone all showed slight inhibition of N^8-acetylspermidine deacetylation activity at 10.0 mM concentrations. Also, naturally occurring compounds such as acetylornithine, acetylcysteine, and acetylglycine showed inhibition only at 10 mM concentrations, and acetylgalactosamine was found not to inhibit this deacetylation activity, even at 10 mM concentrations.

To determine the amount of inhibition of N^8-acetylspermidine deacetylation produced by each compound, Dixon plots (62) were used to estimate K_i for each compound. The Dixon plot is a graph of concentration of inhibitor vs. the reciprocal of the velocity of the reaction (in this case, the velocity of deacetylation). Each line represents a concentration of substrate used (N^8-acetylspermidine concentration). The point where these lines intersect is approximately the negative K_i of that particular reaction being carried out. Figure 15 shows a Dixon plot with N^1-acetylspermidine as the inhibitor, which was found to have the lowest K_i value of all the compounds tested. The results obtained with acetylprocainamide are shown in Figure 16, and this is the only drug tested which showed a K_i below 2.0mM. Table I lists all compounds tested which showed K_i values lower than 2.0mM. Each of the K_i values was determined graphically from Dixon plots as shown for N^1-acetylspermidine in Figure 15. It should be pointed out that only the diamines and polyamines were seen to inhibit N^8-acetylspermidine deacetylation activity with values for the K_i less than 2.0mM.

DISCUSSION

The present study is an attempt to characterize the deacetylating enzyme activity from rat liver cytosol for the deacetylation of N^8-acetylspermidine. The K_m for deacetylation of N^8-acetylspermidine is estimated to be 4.4 μM as determined from the double-reciprocal plot in Figure 6. Libby (40) reported that the partially purified deacetylating enzyme which he studied showed a K_m for N^1-acetylspermidine of approximately 3.0 μM. This is quite close to the value reported here of 4.4 μM for N^8-acetylspermidine, and it seems reasonable to assume that the deacetylating activities are one and the same. The fact that N^8-acetylspermidine has a K_m value in the μM range suggests that the deacetylating activity present in rat liver cytosol is quite specific for N^8-acetylspermidine as a substrate. In comparison, N-acetyl- - alanine is reported to have a K_m of 2.5 mM when hydrolyzed by an enzyme located in hog kidney (63), and N-acetylgalactosamine is deacetylated by an enzyme found in bovine liver with a K_m of 10.8 mM (64).

The K_m is a rate constant which represents the relationship between the rate of enzyme-substrate formation and degradation during a reaction (62). Reporting the K_m of a substrate for a particular enzyme-catalyzed reaction is important for a number of reasons: (i) the K_m establishes an approximate value for the intracellular level of the substrate, (ii) since the K_m is constant for a given enzyme and substrate, its value provides a means of comparing and identifying enzymes from different organisms, or from different tissues within the same organism, and (iii) a change in the apparent K_m by the

addition of certain compounds during a reaction can lead to information about possible activators or inhibitors of the enzyme being studied.

A second important aim of this study is to examine the effects of various potential inhibitors on this enzyme activity. An inhibitor is described by Segel (62) as any substance which reduces the velocity of an enzyme-catalyzed reaction. This reduction in velocity is manifested on a double-reciprocal plot (1/v $\underline{vs}$. 1/$[s]$) as a new line with an increase in slope, and which pivots counterclockwise about its point of intersection with the central line. The point at which this new line intercepts the x-axis is the negative 1/K_m apparent, or the negative reciprocal of the newly observed K_m. A further analysis and quantitation of the inhibition can be made with a replot of K_m apparent $\underline{vs}$ concentration of inhibitor. This plot provides a rate constant referred to as the K_i of the inhibitor for the particular reaction being measured, and this K_i is analagous to the K_m of the substrate for a particular reaction. A more practical and accurate method of determining the K_i of an inhibitor than the replot of K_m apparent $\underline{vs}$. concentration of inhibitor is the Dixon plot (64) which is used in this report. Using several concentrations of inhibitor, a Dixon plot displays 1/v $\underline{vs}$ $[I]$ and the negative K_i is read directly from the value on the x-axis which corresponds to the point where the lines intersect. The Dixon plot can be used even if the nature of the inhibition is unknown ($\underline{i.e.}$, competitive, non-competitive, or mixed) and if the maximum velocity of the reaction (V_{max}) is also unknown.

The present study showed that various compounds were able to inhibit the deacetylation of N^8-acetylspermidine when added to the standard incubation mixture. Only N^1-acetylspermidine was seen to inhibit this deacetylation competitively, as shown in Figure 7 by the intersection of the lines

at the y-axis. This competitive inhibition would suggest that both N^1- and N^8-acetylspermidine are deacetylated by the same enzyme activity. However, the value for the K_i of N^1-acetylspermidine is approximately 160 uM, which is nearly 40 times the K_m of N^8-acetylspermidine reported here. This finding would tend to suggest that N^8-acetylspermidine is the preferred substrate among the compounds tested in this system. Libby (40) reported the K_m of 3.0 μM for N^1-acetylspermidine using a partially purified enzyme preparation from rat liver. The difference in purity of these enzyme systems may account for the differences in these two studies. It is also important to point out that Blankenship (42) reported that N^1-acetylspermidine does not undergo deacetylation as a major metabolic route as does N^8-acetylspermidine when using a crude cytosol preparation. Since N^1-acetylspermidine is known to undergo oxidative cleavage, it is possible that oxidazing enzymes such as polyamine oxidase may be present in the 100,000g fraction of liver used here. It therefore seems reasonable that although the inhibition by N^1-acetylspermidine is competitive, the approximate K_i of N^1-acetylspermidine in this crude cytosol preparation is larger than in a partially purified system due to other major pathways of metabolism which are present.

The parent polyamine compounds showed the greatest inhibition of N^8-acetylspermidine deacetylation aside from N^1-acetylspermidine. Spermidine, spermine, and putrescine inhibited this deacetylation reaction although the type of inhibition (i.e., competitive or mixed) was not determined. An interesting point is that spermidine was found to be the most potent inhibitor of N^8-acetylspermidine deacetylation of these three polyamines with a K_i of 310 μM, followed by spermine at 1.15 mM, and putrescine at 1.45 mM (Table I). This differs from the report by Libby (40) in which spermine was found to be the most potent inhibitor

of N^1-acetylspermidine deacetylation followed by spermidine and putrescine. Again it must be emphasized that these polyamines were added to different enzyme systems when comparing this study to the study by Libby (40).

In addition to putrescine, three other diamino compounds, 1,3-diaminopropane, cadaverine and 1,6-diaminohexane were shown to inhibit the deacetylation of N^8-acetylspermidine, but not to as great an extent as did putrescine. Also, 1,3-diaminopropane inhibited the reaction only slightly unless used at a concentration of 10.0 mM. The K_i for both cadaverine and 1,6-diaminohexane are approximately the same at 1.5 mM. These results suggest that the length of the carbon change between amino groups is one factor affecting the affinity of a compound for the deacetylating enzyme, along with other factors such as number of amino groups on the molecule. This may explain why spermidine and spermine have lower K_i values than putrescine or other diamines tested.

Acetylprocainamide was the only drug found to inhibit N^8-acetylspermidine deacetylation, with a K_i of less than 2.0 mM (approximately 1.9 mM from Figure 16). There is no available literature which discusses the _in vivo_ deacetylation of this compound, and it is therefore very difficult to speculate on the enzyme(s) normally involved in the deacetylation of acetylprocainamide. One factor to consider is that a concentration of nearly 1.9 mM acetylprocainamide would have to be present in the soluble fraction of the liver before the deacetylation activity would metabolize a significant amount of acetylprocainamide. Moreover, it must be pointed out that the structure of acetylprocainamide contains three amino groups--two of which are in an aliphatic portion of the molecule separated by two carbon atoms. This structure may play a role in inhibiting this enzyme activity. Certainly it is not possible to determine from the present inhibition studies whether acetylprocainamide

can actually serve as a substrate for this deacetylating enzyme.

Aspirin was the only other compound tested which inhibited N^8-acetylspermidine deacetylation activity at concentrations less than 10.0 mM. However, this inhibition is probably due to aspirin lowering the pH of the final standard incubation mixture, which in turn may decrease the deacetylating activity. This finding is consistent with the findings of Blankenship (41) that deacetylation activity of N^8-acetylspermidine is reduced when the pH of the standard incubation mixture is dropped below 7.3. Other compounds were shown either to inhibit deacetylation activity of N^8-acetylspermidine slightly or not at all. Acetylacetone was expected and was found to be inhibiting the N^8-acetylspermidine deacetylation activity since its hydrolysis involves the breaking of an ester bond rather than an amide bond. Neostigmine is known to be an inhibitor of esterases, especially acetylcholinesterase (65), and was also found to inhibit the observed deacetylation activity only slightly. The major deacetylating enzymes for phenacetin are found in the microsomes (55), and the observation here that phenacetin is not effective in inhibiting N^8-acetylspermidine deacetylation is consistent with this finding. Colchicine and acetylgalactosamine, both of which are known to be deacetylated in the liver (57,64) also showed no inhibition of this deacetylation activity which metabolizes N^8-acetylspermidine.

An interesting observation made in the present report is that acetanilid and the acetylated amino acids, acetylglycine and acetylcysteine, each produced only slight inhibition of N^8-acetylspermidine deacetylation at 10.0 mM concentrations. Since Bray (48) showed that the amidohydrolases which metabolize acetanilid and the acetylated amino acids are different, these present data indicate the presence of an additional amidohydrolase enzyme activity which deacetylates N^8-acetylspermidine

and is different from the enzymes which deacetylate acetanilid and the acetylated amino acids. This is especially interesting since acetanilid deacetylation is known to occur primarily in the soluble fraction of the liver (52). The fact that neither acetylated amino acids nor acetyl-galactosamine caused an inhibition of N^8-acetylspermidine deacetylation activity agrees with the deacetylation data for N^1-acetylspermidine reported by Libby (40).

Finally, two acetylated compounds which have not been studied as to their routes of deacetylation in mammalian systems are acetylpenicillamine and acetylornithine. Both of these compounds showed only slight inhibition of N^8-acetylspermidine deacetylation activity at high concentration. It is of interest that acetylornithine is the acetylated precursor of putrescine but showed no inhibition of deacetylating activity at concentrations less than 10.0 mM.

The above data indicate that the enzyme activity from rat liver which deacetylates N^8-acetylspermidine is different from many other deacetylating activities known, including amidohydrolases which metabolize endogenous compounds as well as enzymes which deacetylate exogenous compounds. This observation along with findings that the K_m for N^8-acetyl-spermidine in this deacetylation reaction is in the uM range suggests that the enzyme may be specific for N^8-acetylspermidine and other acetylated polyamines as substrates for deacetylation. A possible function of this N^8-acetylspermidine deacetylation is to regulate the intracellular levels of polyamines. The acetylated polyamines which are produced in the nucleus by an acetyltransferase can become deacetylated in the cytosol by an amidohydrolase (41). The finding that non-acetylated polyamine compounds show an inhibition of this deacetylating activity allows for the possibility of a feedback inhibition by the product of the reaction,

which could shut down the N^8-acetylspermidine deacetylating activity when the appropriate concentration of spermidine was reached. Further, investigation is needed to confirm or disprove this proposed role for acetylpolyamine deacetylation.

CONCLUSIONS

An apparent K_m of 4.4 μM was determined for the deacetylation of N^8-acetylspermidine by the soluble fraction of rat liver. This suggests a relatively high degree of specificity for N^8-acetylspermidine and possibly other acetylated polyamines by this deacetylating activity.

N^1-Acetylspermidine was the only compound used in this study which clearly showed a pure competitive antagonism of the observed N^8-acetylspermidine deacetylation activity. Of all the compounds which were found to inhibit N^8-acetylspermidine deacetylation, N^1-acetylspermidine was the most potent inhibitor with a K_i in this system of 160 μM.

Various diamine and polyamine compounds were also shown to inhibit N^8-acetylspermidine deacetylation activity in this study. Of the naturally occurring polyamines, spermidine was found to be the most potent inhibitor, followed in decreasing order of potency by spermine, putrescine, and cadaverine.

Acetylprocainamide inhibited the N^8-acetylspermidine deacetylation at relatively low concentrations and was found to have a K_i comparable to those of the diamines. Other acetylated compounds (including acetylated amino acids, acetylaminosugars, and acetylated aromatic amines) showed no significant inhibition of the deacetylating activity in the range of concentrations studied.

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