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GLYOXAL AND ESTRADIOL MEDIATED ALTERATIONS IN RAT UTERINE HISTONE ACETYLATION

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GLYOXAL AND ESTRADIOL MEDIATED ALTERATIONS IN RAT UTERINE HISTONE ACETYLATION

BY

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ABSTRACT

Procaccini, Robert Louis. Ph. D., University of Rhode Island, June, 1972. Glyoxal and Estradiol Mediated Alterations in Rat Uterine Histone Acetylation. Major Professor: Dr. David R. DeFanti.

The immature rat uterus was developed as a model in which to measure alterations in histone acetylation in a target organ following hormonal stimulation. The effect of various glyoxal derivatives on histone acetylation and several other parameters was measured in the uterine tissue of untreated and estradiol treated animals. Six day treatment with glyoxal monohydrate and methylglyoxal-bis-guanyl-hydrazone (Methyl-GAG) decreased uterine wet weight and partially blocked estradiol mediated alterations in uterine wet weight in animals receiving concomitant estradiol and glyoxal treatment.

The dose dependent in vitro inhibition of uterine histone acetylation is thought to occur through direct action on the acetylation system. The I_{50} values reported for methylglyoxal and phenylglyoxal suggest that the drugs are equally effective as inhibitors of uterine histone acetylation.

Estradiol mediated alterations in uterine histone acetylation were characterized as a rapid depression of enzyme activity; 24 hours following hormone treatment this enzyme activity was elevated above control levels. The elevation in histone acetylation following estradiol treatment was blocked by either methylglyoxal or phenylglyoxal administered 20 hours after estradiol. Uterine wet weight, total DNA,

RNA/DNA and protein/DNA ratios were not affected by this treatment. These data suggest that histone acetylation may not be of primary importance in estradiol stimulation of the rat uterus.

The phenylglyoxal mediated depression of uterine histone acetylation preceded the alterations in RNA/DNA and protein/DNA ratios observed when phenylglyoxal pretreatment was extended to 48 hours. Elevations in total DNA and protein/DNA ratios in the uterus of estradiol treated rats were partially suppressed in animals pretreated with phenylglyoxal, whereas the RNA/DNA ratio was not affected in these animals.

Results from this study suggest that the glyoxal mediated blockade of uterine histone acetylation may lead to an alteration in nucleic acid content of the hormonally stimulated uterus.

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I would like to express my deep appreciation to Dr. David R. DeFanti, Dr. John J. DeFeo and Dr. George Osborne for their continued help and encouragement throughout my graduate education.

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DEDICATION

To my wife, Ruth Ann, and to my parents, for their endless patience and encouragement.

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I. INTRODUCTION

The biological activity of glyoxal derivatives (ketoaldehydes) has been the subject of numerous literature reports. In 1967, Szent-Gyorgyi proposed that these compounds may inhibit cell growth. Interestingly, methylglyoxal and propylglyoxal were shown to inhibit cell division in mouse lymphoma cells (Gregg, 1968). Klamerth (1968) suggested that glyoxal may elicit its cellular inhibitory properties at the DNA replication level. Scaife (1969) has presented evidence that β-ethoxy-α-ketobutyraldehyde (Kethoxal, Upjohn Pharmaceutical) inhibits cell division and protein synthesis. Although kethoxal and another ketoaldehyde, methylglyoxal-bis-guanylhydrazone, have been used therapeutically for leukemia, the exact antileukemic mechanism is not known (Henderson, 1969).

Concomitantly, a growing body of observations (Allfrey et al., 1963; Pogo et al., 1966, 1968) indicates that histone metabolism, in particular, histone acetylation, may be involved in regulating DNA function. The inhibitory action of histones on cellular processes may itself be regulated, in part, by the acetylation reaction (Georgiev, 1969; Tsanov and Sendov, 1971). It follows that inhibition of histone acetylation may lead to alterations in overall histone function. In addition, previous workers have reported (Pogo et al., 1966) alterations in histone acetylation following hormonal stimulation.

As a result, this investigation was designed to study the effects of glyoxal derivatives on histone acetylation in a hormonally alterable system.

II. LITERATURE SURVEY

During the past two decades, numerous investigators have tried to elucidate the structure, mechanism of action, and general cellular function of histones. Because these low molecular weight, highly alkaline proteins are closely associated with deoxyribonucleic acid in the nuclear apparatus of cells, early investigators focused their attention on the search for proof of a regulatory function involving the histones.

The first hypothesis was presented by Stedman and Stedman (1950) that histones were inhibitors of genetic activity. Later, the suggestion was made that the evolution of the structure and function of various histone species parallels, to some degree, the evolution of the genes themselves. Georgiev (1969) stated that histones appeared in evolution at the same time the cell acquired two of its fundamental properties: a nuclear membrane and the capacity for differentiation. DeLange and Smith (1971) pointed out that evolutionary conservatism is evident in the structure of histones. Histone IV (arginine-rich), with an approximate evolutionary mutation rate of 0.06/100 residues/ 100 million years, is 33 times more genetically stable than any known protein. It has been proposed that the biological function of the histones demands such close control of its structure that even slight modifications in its amino acid sequence could not be tolerated (Bradbury, 1969).

STRUCTURE AND FUNCTION OF HISTONES

Approximately 15 percent of the nuclear component of eukaryotic cells is composed of histone protein. The histones are believed to exist in five distinct fractions: f1, lysine-rich; f2b, moderately lysine-rich; and f2a1, f2a2, and f3, arginine-rich histones. Each of the fractions is thought to comprise 20 percent of the whole histone (Richards and Pardon, 1970). Virtually all of the deoxyribonucleic acid (DNA) in the nucleus exists as a complex with histones. It is generally believed that histones attach to DNA through electrostatic linkages. Of these, the strongest are ionic linkages between the phosphate groups of the DNA and the basic residues lysine (ϵ ammonium), arginine (guanidinium), and histidine (Richards and Pardon, 1970; Lewin, 1970). Combard and Venduly (1970) presented evidence indicating that different histone fractions interact with DNA at different sites on the DNA molecule. They found lysine-rich histones in association with DNA in regions of relatively high adenine-thymine base content, whereas arginine-rich histones were found in association with guanine and cytosine-rich regions.

Not all the histone fractions affect the activity of the DNA complex to the same extent; for example, lysine-rich histone (f1) inhibits the DNA-dependent-RNA polymerase reaction to a lesser extent than does the arginine-rich histone (f3). Further, prior acetylation of uterine histone markedly depresses the inhibitory effect of

histones on in vitro RNA synthesis (Teng and Hamilton, 1969). The inhibitory effect of arginine-rich histone fractions on DNA dependent-RNA polymerase is thought to be related to the thiol/disulfide ratio of the fraction. Hilton and Stockton (1966) found that diffuse, active chromatin has a greater proportion of sulfur residues as free thiol groups than does dense, inactive chromatin. Purified f3 histone was annealed with DNA, and purified RNA polymerase was used to measure the efficiency of the treated DNA as a template. When histone thiol groups were oxidized to the disulfide form before the annealing step, histone-histone aggregates were produced which possessed augmented inhibitory properties against DNA template activity. These results suggested that continuous RNA synthesis in mammalian cells may be partly dependent upon the existence of cysteine-containing histones in their thiol form (Ord and Stocken, 1969).

Extensive studies of histone metabolism in mammalian cells indicate that these proteins undergo structural alterations by at least three enzymatic processes: (1) acetylation (Allfrey et al., 1964; Pogo et al., 1966; Pogo et al., 1968), (2) methylation (Paik and Kim, 1969; Paik and Kim, 1971), and (3) phosphorylation (Langan, 1969).

The enzymatic modification of histone protein may represent an early event in the complex process of gene activation for subsequent RNA and protein synthesis (Pogo et al., 1968). To support this theory, several model systems have been proposed for the study of histones

and their enzymatic modification during periods of increased growth believed to involve enhanced genetic activity.

Incorporation studies have established that transformation (activation) of human lymphocytes by phytohemagglutinin involves an increase in RNA synthesis (Rubin and Cooper, 1963) and in protein synthesis (Bach and Hirschorn, 1963), using uridine-2-^{14}C and alanine-1-^{14}C, respectively. Increased histone acetylation seen in response to phytohemagglutinin preceded the increase in RNA and protein synthesis; at the same time, cells not stimulated by phytohemagglutinin did not demonstrate this increase in acetylation of their histones (Pogo et al., 1966).

Pogo et al. (1968), studying rat liver regeneration, showed that turnover of ^{3}H-acetyl groups incorporated into histones decreased in partially hepatectomized rats. Rates of ^{3}H-UMP incorporation into RNA reached maximum levels only after histone acetylation had peaked. Pogo (1969) found that nucleolar activation in this system began at 2 hours and chromatin activation at about 6 to 8 hours after partial hepatectomy. He also found that arginine-rich (f2a1) histones were highly and rapidly acetylated. When the acetylated histones were treated with 2M hydroxylamine for one hour, all the labeled acetate was found to be resistant to cleavage from histone. Since hydroxylamine, under these conditions, will hydrolyze O-acetyl but not N-acetyl linkages, and since, under similar conditions, the histone fraction f3

(arginine-rich) lost 55 percent of its acetyl content, the presence of O-acetylated groups was indicated. Verifications of the presence of radioactive acetyl groups was accomplished by acid hydrolysis and steam distillation of volatile acetic acid.

An extensive study by Paik and Kim (1971) has shown that at least three enzymes found in the cytosol fraction of calf thymus are capable of methylating histones. Protein methylase I(S-adenosyl-L-methionine: protein-arginine methyltransferase) methylates the guanido group of arginine residues in histones yielding a product identified as ω -N-methylarginine. When protein methylase I was measured in rapidly growing hepatoma, the enzyme activity was found to increase by 50 to 100 percent (Paik et al., in press). Kaye and Sheratyky (1969) obtained an enzyme preparation from the 105,000 x g supernatant fraction of rat organs which they found capable of transferring methyl groups from S-adenasyl-L-methionine and acetyl groups from acetyl-COA to arginyl and lysyl residues in histones. The most effective acceptors were unfractionated histones of rat organs, calf thymus arginine-rich histones and poly-L-arginine.

An enzyme system derived from rat brain nuclei selectively catalyzed the acetylation of histones, using acetyl-COA as acetate donor (Bondy et al., 1970). The preparation was most active in acetylating arginine-rich histones. Histone acetylase prepared from nuclei of adult rat brains exhibited greater activity than did similar

preparations obtained from the brains of newborn rats. In contrast, brain cytoplasmic enzyme was low in both specificity and activity. Treatment of cerebral chromatin with a solubilized acetylase preparation augmented the template activity of the treated chromatin for RNA synthesis.

Non-enzymatic acetylation of histones has been reported by Paik et al. (1970); however, the reaction occurs almost exclusively in nuclei and has a pH maximum of 10.0.

Imoue and Fujimoto (1969) report a histone deacetylating enzyme present in calf thymus extracts. The significance of this enzyme in biological systems, however, is not known.

It is evident from these studies that histone modification is an active process occurring most significantly during periods of enhanced cell growth. Measurement in several systems indicates that histone modification, particularly acetylation, precedes or accompanies increases in DNA-dependent-RNA polymerase activity and RNA synthesis in stimulated cells (Wilhelm and McCarthy, 1970). However, neither direct proof for the involvement of histones nor their modification in the control of RNA synthesis is yet available (Georgiev, 1969; DeLange and Smith, 1971).

HISTONES AND NUCLEAR MECHANISMS

Butler (1966) proposed a mechanism for the regulation of gene activity involving an interaction between hormone, target protein, and

histone. According to his model, the hormone, upon reaching its target organ, would be specifically and preferentially bound to a target cell protein with subsequent formation of a protein-hormone complex. This complex would then attach or somehow interact with DNA-associated histone and either remove the histone from the nucleoprotein complex or diminish the degree of binding between DNA and histone. The now derepressed DNA segment would be free to take part in nuclear transcriptional functions. This model becomes most plausible when viewed in light of recent findings that various steroid hormones are capable of increasing DNA template activity, RNA synthesis, and protein synthesis in their respective target organs (Hamilton, 1968; Kenney et al., 1968; Avdalovic and Kochakian, 1969). The biological activity of at least one steroid hormone, estradiol-17-β, is believed to be initiated by the attachment of hormone to a cytoplasmic "receptor" protein, followed by translocation of the hormone-protein complex into the cell nucleus (Jensen et al., 1968; Mueller, 1971; Truong and Baulieu, 1971). The early actions of this hormone on intact immature and ovariectomized rat uterus is well documented (Teng and Hamilton, 1969; Bresnich, 1971; and Mueller, 1971). It is generally accepted that the action of estradiol on its target organ involves, in part, an activation of nuclear mechanisms for genetic expression (Mueller, 1971). Estradiol may directly affect the template activity of uterine deoxyribonucleoprotein, which is firmly attached to RNA polymerase.

This action is thought to explain the observed increase in RNA synthesis without an apparent increase in RNA polymerase following estradiol administration (Gorski et al., 1965).

From 0-24 hours following estradiol treatment, synthesis of uterine histones remain unchanged. However, at 12-24 hours following hormone treatment, the rate of chromatin-directed RNA synthesis in vitro decreases, whereas RNA levels increase during the latter stages of estrogen action. From these observations, Gorski et al. (1965) suggest that estradiol may alter the metabolism of uterine RNA.

Teng and Hamilton (1969), investigating the involvement of histones in estrogenic action recently demonstrated that uterine histones, particularly arginine-rich fractions, are capable of inhibiting uterine chromatin-directed RNA synthesis, in vitro. In vivo stimulation of template activity by estradiol-protected RNA synthesis in vitro from the inhibitory effects of added histone. Estradiol, added in vitro at final concentrations of 10^{-5} - 10^{-6} M, did not protect uterine chromatin-directed RNA synthesis from inhibition by the histones.

The amount of arginine-rich histones in association with uterine chromatin decreases during the first hour of estradiol administration (Barker, 1971). It is not known whether the histones are metabolized through protolysis or are complexed with non-histone protein. The presence of a specific enzyme capable of degrading histones (histone hydrolase) has been reported in an amphibian system (Paik, 1971).

Libby (1968) reported the presence of a soluble enzyme present in immature rat uterus, which is capable of acetylating histone. In this study, addition of estradiol in vitro stimulated histone acetylation. The ability of estradiol to stimulate histone acetylation in vivo during the first 15 minutes of hormone treatment has been demonstrated by Libby (1971). However, Anderson and Gorski (1971) reported that, after introduction of estradiol, histone acetylation was at first depressed but then rose, beginning at about 12 hours and elevating above control levels at 24 hours following hormone treatment. In their studies with protein methylase, Kaye and Sheratzky (1969) found that estradiol-17-β, preincubated with a 150,000 x g supernatant fraction of immature rat uterus and then added to a standard assay, decreased the methylation of histone by 10 percent. Furthermore, estradiol-17-β, administered 50 μg/rat, subcutaneously, for 3 days decreased histone methylation by 50 percent, measured in vitro.

Steroid hormones are capable of direct attachment to histones both in vitro and in vivo (Sluyser, 1966; Sunago and Koide, 1967; Sluyser, 1970). The greatest extent of hormonal interaction with histones occurs with arginine-rich histone fractions. The effects of such interactions could be considerable, since histone attachment to DNA may involve lysyl and arginyl histone residues (Lewin, 1970).

The interaction between cortisol-1,2-t and histones is thought to be dependent on the presence of 21-dehydrocortisol, a contaminant in

most corticosteroid preparations and the possible active metabolite of cortisol in vivo (DeLange and Smith, 1971). In studies performed by Mander and Walker (1970), cortisol and 21-dehydrocortisol, a steroid with a glyoxal moiety in its structure, reacted with all histone fractions tested in vitro. However, both steroids were bound most extensively to arginine-rich histones. Blockade of arginyl histone residues by gloxylation (Freedman et al., 1968) greatly diminished binding of 21-dehydrocortisol and eliminated binding of cortisol to the histones. In addition, the results indicate that corticosteroid binding to histones may require prior oxidation of the steroid. The actual binding site may involve covalent interactions between the steroids and residues of the basic amino acids, arginine and lysine, in histone protein. However, the biological significance of these interactions has not been assessed.

GLYOXALS AND THEIR CELLULAR MECHANISMS

Glyoxals are ketoaldehydes closely related to trioses and other basic metabolites. They reportedly exist endogenously in high concentrations (Szent-Gyorgyi, 1967). Examples of endogenous metabolism leading to the formation of glyoxals include glycine to methylglyoxal and ascorbic acid oxidation to dehydroascorbate (Edgar, 1969). The latter compound contains a glyoxal moiety in its structure and reportedly possesses antitumor properties (Edgar, 1969). Methylglyoxal is metabolized to lactic acid by two closely related enzymes,

glyoxylase I and glyoxylase II (French and Freelander, 1958). Vince and Wass (1969) are exploring the possibility of inhibiting the glyoxylase system in order to potentiate the carcinostatic activity of methylglyoxal.

Szent-Gyorgyi (1968) has proposed that endogenous inhibitors of cell division may include molecular species containing a glyoxal group (R - C - C - H). In advance of this proposal, Szent-Gyorgyi stated that cells possess an unlimited source of metabolically available electrons but are deficient in electron acceptors. Sulfhydryl groups involved in the complex mechanisms of cell proliferation and protein synthesis would exist in their thiol form and could be oxidized to the disulfide form by glyoxals and thereby be inactivated. In subsequent studies, the glyoxals, particularly methylglyoxal and propylglyoxal, inhibited cell division in both bacterial and mammalian cells (ascites tumor) at concentrations of 10^{-3}M (Egyud and Szent-Gyorgyi, 1966). Incorporation studies demonstrated that, although protein synthesis was blocked to a greater extent, both DNA and RNA synthesis was inhibited by the glyoxals (Egyud and Szent-Gyorgyi, 1966).

Methylglyoxal and propylglyoxal inhibit cell division in mouse lymphoma (L-5178Y) by their ability to block protein synthesis (Gregg, 1968), whereas glyoxal inhibits DNA synthesis as well as protein synthesis in human fibroblasts (Klamerth, 1968). Scaife (1969) measured ^{3}H-uridine incorporation into RNA and ^{3}H-thymidine

incorporation into DNA in human kidney cells grown in monolayer culture and treated with methylglyoxal or Kethoxal (β-ethoxy-α-keto-butyraldehyde). Although DNA, RNA, and protein synthesis were inhibited, the most pronounced effect of these compounds was the blockade of DNA and protein synthesis.

Procaccini et al. (1971) used a soluble rat uterine enzyme to study the inhibition by methylglyoxal and phenylglyoxal of histone acetylation in an in vitro system. They postulated that, if such an inhibitory effect is accomplished in vivo, it might offer an alternate/additional explanation to the known inhibitory properties of the glyoxals on cell division and proliferation.

Interestingly, two glyoxal derivatives, methylglyoxal-bis-guanylhydrazone (Methyl-GAG) and Kethoxal have found limited use in the treatment of leukemia (Freireich et al., 1962; French and Freelander, 1958). However, the exact mechanism of action of these drugs is not yet known (Henderson, 1969).

III. EXPERIMENTAL

A. ANIMALS

Immature (22-30 day old) female rats (Sprague-Dawley strain; Charles River Breeding Laboratories, Wilmington, Massachusetts) were housed in quarters maintained at 70°C supplied with 12-hour alternating cycle of light and dark. Animals were offered food (Purina Rat Chow, Ralston Purina Company, St. Louis, Missouri) and water, ad libitum. However, food was removed 24 hours prior to sacrifice in the case of 24-hour time-course studies.

B. SUBCELLULAR FRACTIONATION AND LOCALIZATION OF HISTONE ACETYLATING ACTIVITY: SPECIFIC ACTIVITY

Eight immature (25 day old) female rats were sacrificed by cervical dislocation. The uterine tissue was cleaned of connective tissue, in situ, excised, blotted and weighed. The pooled tissues were homogenized in 9 volumes of 0.25M cold sucrose solution using a Tri-R homogenizer (Tri-R-Instruments, Rockville Centre, New York). The homogenated were pooled, separated into 15 ml Nalgene centrifuge tubes and centrifuged at 780 x g (2,500 RPM) for 10 minutes at 0°C in a Servall refrigerated centrifuge (Sorvall, Inc., Norwalk, Connecticut). The supernatant was transferred to another set of tubes and centrifuged at 10,000 x g (9,500 RPM) for 15 minutes. The nuclear fraction was resuspended to volume in 0.25M sucrose solution and centrifuged

as before. The resultant supernatant was decanted and discarded, the nuclear pellet was resuspended to volume with Robinson's media (Robinson, 1949), placed in glass screw-cap vials and frozen at -40°C until required for assay. The 10,000 x g supernatant fraction was transferred to straight-walled plastic centrifuge tubes, capped and centrifuged at 105,000 x g (32,000 RPM) for 1 hour at 0°C in an IEC Preparation Ultracentrifuge (Model B-60, International Equipment Company, Needham Heights, Massachusetts) equipped with an A-170 aluminum head. The mitochondrial fraction was resuspended to volume in 0.25 M sucrose solution and centrifuged at 10,000 x g as before. The resultant supernatant was discarded. The mitochondrial fraction was resuspended to volume in Robinson's media, transferred to screw-cap vials and stored at -40°C until required for assay. The 105,000 x g supernatant fraction was transferred to storage vials and frozen as above. The microsomes were washed twice with 0.25 M sucrose solution, resuspended to volume in Robinson's media, and stored at -40°C until needed for assay. An aliquot of the thawed fraction required for assay of histone acetylation activity was used for protein determinations on all subcellular fractions.

C. IN VITRO STUDIES

1. Temperature study: Determination of temperature coefficient (Q_{10}). Components of the complete assay system previously described were incubated in a Dubnoff metabolic incubator shaker (Dubnoff,

Precision Scientific Company, Chicago, Illinois) set at 30°, 37°, 40°, 45°, 50°, 55°, and 60°C for 30 minutes. Histone acetylating activity at the various temperatures was determined. These data were inserted into the van't Hoff equation to determine the Q_{10} value:

$$\log Q_{10} = \frac{10}{t_2 - t_1} \log \frac{k_2}{k_1}$$

where: k_2 = reaction rate at temperature t_2

and k_1 = reaction rate at temperature t_1

2. Dose response for methyl- and phenylglyoxal: determination of I_{50}. Phenylglyoxal (Pfalz and Bauer) and methylglyoxal (Aldrich Chemical) were dissolved in 0.5 M phosphate buffer, pH 7.7 prior to use. Various concentrations of drugs were preincubated in the presence of enzyme in the assay medium at 37°C for 15 minutes; histone was added, and the reaction was allowed to proceed for an additional 30 minutes. The data were plotted (% inhibition vs. drug concentration), and the I_{50} value (drug concentration at which 50 percent inhibition is observed) was determined graphically.

3. Effect of enzyme-drug and histone-drug preincubation on histone acetylation. (a) Enzyme-Drug: Phenylglyoxal was dissolved in 0.05 M phosphate buffer pH 7.7, to a concentration of 10^{-2} M. From this stock solution, serial dilutions of 10^{-3} M and 10^{-4} M phenylglyoxal were prepared. A 0.25 ml aliquot of each concentration of phenylglyoxal was transferred to each of three tubes containing 0.25 ml of 10,000 x g

uterine supernatant fraction in concentrations of 5×10^{-3} M, 5×10^{-4} M, and 5×10^{-5} M phenylglyoxal, respectively. The contents of the tubes were mixed and incubated at 37°C. At the end of a 10 minute pre-incubation period, a 0.1 ml aliquot of each mixture was transferred to tubes containing all components of the standard assay system with exception of uterine supernatant. The tube contents were mixed and incubated, with shaking, for 20 minutes at 37°C. Uterine supernatant, preincubated with phosphate buffer constituted the control samples. Standard procedures for the determination of histone acetylation activity were performed on each sample.

(b) Histone-Drug: Solutions of phenylglyoxal at concentrations of 10^{-2} M, 10^{-3} M and 10^{-4} M were prepared as before. A 0.6 ml aliquot of a freshly prepared solution of calf thymus histone (10 mg/ml) was added to tubes containing 0.6 ml of phenylglyoxal solution at various concentrations specified. The resultant mixtures, containing phenylglyoxal at concentrations of 5×10^{-3} M, 5×10^{-4} M and 5×10^{-5} M, respectively, were incubated, with shaking, for 10 minutes at 37°C. Following this period, a 0.2 ml aliquot containing 1.0 mg of histone required for assay was transferred to tubes containing all components of the assay system with the exception of histone. The completed mixture was then incubated for an additional 20 minutes at 37°C with shaking. Histone, preincubated with buffer, constituted the controls. Assay of histone acetylating activity of the various samples was accomplished as above.

D. IN VIVO STUDIES

1. Tissue Weight: Alterations with Estradiol and Glyoxals. Immature female rats (26 days old), weighing 70-90 grams, were divided into groups of five animals each. Groups were housed in plastic box cages furnished with wood chip bedding. Animals were allowed food and water ad libitum. Solutions of drugs in 0.05M phosphate buffer (pH 7.4) were prepared fresh daily and administered according to the following schedule:

(1) phosphate buffer, 1 ml/kg, intraperitoneally, for 6 days, plus 0.2 ml saline subcutaneously, every 12 hours for 60 hours, starting on day 3 of schedule.

(2) phosphate buffer, 1 ml/kg, intraperitoneally, for 6 days, plus estradiol-17-β dipropionate (Mann Research Labs) 0.2 ml (12 ug) subcutaneously, every 12 hours for 60 hours, starting on day 3 of schedule.

(3) glyoxal monohydrate, 200 mg/kg, intraperitoneally, daily for 6 days.

(4) glyoxal monohydrate, 200 mg/kg, intraperitoneally, daily for 6 days, plus estradiol, 0.2 ml, subcutaneously, every 12 hours for 60 hours, starting on day 3 of schedule.

(5) methylglyoxal-guanylhydrazone (methyl-GAG, Aldrich Chemical) 20 mg/kg, intraperitoneally, daily for 6 days.

(6) methylglyoxal-guanylhydrazone, 20 mg/kg, intraperitoneally, daily for 6 days, plus estradiol, 0.2 ml, subcutaneously, every 12 hours for 60 hours, starting on day 3 of schedule.

Rats were sacrificed by cervical dislocation. The liver of each animal was perfused in situ with cold 0.25 M sucrose solution, removed, weighed, and placed on ice. The uterus was surgically cleaned of connective tissue, excised, press blotted and weighed. The liver and uterus from each animal was homogenized separately in 9 volumes of Robinson's medium (Robinson, 1949), using a Polytron homogenizer (Speed #6 for 15 sec.), followed by centrifugation at 10,000 x g (9,500 RPM) for 10 minutes at 0°C in a Servall Refrigerated Centrifuge (Sorval, Model RC2-B). The supernatant fraction of liver and uterus was transferred in 1.0 ml aliquots to 5 ml glass screw-cap vials and stored at -40°C until required for enzymatic assay.

2. Estradiol and Phenylglyoxal: Initial response. A stock solution of estradiol-17-β (Scwartz-Mann) was prepared by dissolving 25 mg of the hormone in 100 ml of 95% ethyl alcohol. At the time of administration, 1.0 ml of ethanolic stock solution was slowly added to 3.0 ml of saline with constant stirring to prepare a suspension containing 60 μg/ml of estradiol. At the time of injection (treatment 2) each rat received approximately 12 μg of estradiol (0.2 ml) subcutaneously. A freshly prepared solution of phenylglyoxal monohydrate in 0.05 M phosphate buffer was adjusted to pH 7.4 with 0.5N NaOH.

Four groups of eight, 26-day old female rats were administered phenylglyoxal, 250 mg/kg, intraperitoneally (Treatment 1). At the end of one hour, each animal received estradiol, 12 µg/rat, subcutaneously (Treatment 2). Groups receiving estradiol, following phenylglyoxal pretreatment or estradiol alone, were sacrificed at 0, 5, 10, and 15 minutes following estradiol treatment. Control animals receiving 1 ml/kg of 0.05M phosphate buffer alone were sacrificed one hour following injection. Rats were sacrificed by cervical dislocation. Individual uterine tissue was press blotted, and weighed, and the pooled tissues from two identically treated animals were homogenized in 9 volumes of cold Robinson's media. The 10,000 x g supernatant fractions were stored at -40°C in 5 ml glass screw-cap vials until required for assay. Protein determinations of the supernatant fractions obtained from experimental groups were carried out by the method of Lowry et al. (1951). Students "t" test was employed to determine statistical differences between treated and untreated groups.

3. Activity of Estradiol and Glyoxals: Time response.

Immature (25 day old) female rats were divided into groups of 6 to 8 animals. Control groups received 1 ml/kg of the drug vehicle, 0.05 M phosphate buffer (pH 7.4) or estradiol vehicle. Rats were administered either estradiol-17-β, 10 ug/rat, intraperitoneally, or phenylglyoxal, 250 mg/kg, intraperitoneally. Animals treated with either drug were sacrificed at 1, 2, 4, 8, 16, and 24 hours following drug administration.

Rats were sacrificed by cervical dislocation. The uterine tissue was surgically cleaned, blotted and weighed. For histone acetylation studies, tissues from two rats were pooled and homogenized in 9 volumes of Robinson's media. Further preparation and storage of the uterine extract for subsequent analysis of histone acetylation activity was as described previously.

4. Drug Interaction: Estradiol and Glyoxals. Thirty day old female rats were divided into groups of 8 to 10 animals. Rats were administered estradiol, 10 ug/rat, intraperitoneally. Control animals received estradiol vehicle as described previously. At 20 hours following hormone treatment, estradiol treated rats received either methylglyoxal (125 mg/kg or 250 mg/kg) or phenylglyoxal (250 mg/kg), intraperitoneally. Vehicle treated rats also received the same doses of the glyoxals. Control rats received 1 ml/kg of the drug vehicle, 0.05 M phosphate buffer (pH 7.4). Groups of animals were injected at appropriate intervals in order to account for the time required for sacrifice of each group. All animals were sacrificed 24 hours after estradiol administration (4 hours following glyoxal administration).

Protocol followed after sacrifice, involving weighing, uterine extract preparation and assay were as described previously except that tissues from this study were not pooled. Protein determinations were carried out by the method of Lowry et al. (1951).

E. ASSAY OF HISTONE ACETYLATION

The procedure for the analysis of histone acetylation is a modification of the method of Libby (1968). A 0.05 ml aliquot of a 10,000 x g supernatant fraction of rat uterus (prepared as previously described) capable of acetylating histones was routinely employed as the enzyme source in the assay system. The assay mixture contained, in addition to the supernatant fraction, 100 umoles of phosphate buffer (pH 7.7), 5 umoles ATP (Sigma Chemical Company) solution adjusted to neutrality with 0.5N NaOH, 0.05 umoles coenzyme A (Cal-Biochem), 5u C Na-acetate-1-^{14}C (New England Nuclear; specific activities of 57-60 millicuries/millimole); and 1.0 mg calf thymus histone (Worthington Biochemicals and enough distilled water to make a final volume of 1.0 ml. Components of the incubation mixture were pipetted into 15 ml Nalgene tubes. All drug preparations were made in the buffer used for enzyme incubations. Incubations were carried out in a Dubnoff metabolic shaker at 37°C under air. Following the prescribed incubation time, the reaction was terminated by the addition of 5 ml of cold acetone. The tubes were sealed with Parafilm (American Can Company, Neenan, Wisconsin), mixed by inversion and allowed to stand in a freezer at -40°C for approximately 30 minutes. The tubes were then centrifuged for 10 minutes at 10,000 x g in a Sorval Model RC2-B refrigerated centrifuge at 0°C. The resultant aqueous-acetone supernatant was decanted and discarded and the tubes inverted and allowed to dry. Drying was usually complete in 30 - 40 minutes. The

precipitate was resuspended in 1.0 ml of 0.2N H_2SO_4 by mixing in a Vortex mixer for 10 seconds, and the tubes were allowed to stand at room temperature for 20 minutes. The tubes were then centrifuged at 10,000 x g for 10 minutes and the resultant supernatant was carefully decanted into another set of Nalgene tubes. After the addition of 5 ml of cold acetone, the tubes were mixed by inversion, as before, and allowed to stand in a freezer for approximately 30 minutes. The tubes were centrifuged for 10 minutes at 10,000 x g and the supernatant discarded. One ml of 0.05 M barbital buffer, 7.5 M in urea (pH 9.0), was added to the precipitate and mixed on a Vortex mixer for 10 seconds. The tubes were allowed to stand at room temperature for 15-20 minutes at which time 0.5 ml of this solution was transferred to glass counting vials containing 15 ml of a scintillation cocktail which was prepared by dissolving 4 g of 2-5-diphenyloxazole (PPO) plus 50 mg of 1,4-phenylene-bio-2-(5-phenyloxazole) in 700 ml of toluene plus 300 ml of BBS-3 (Bio-Solv, Beckman Instruments, Inc.). The vials were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer at a counting efficiency of approximately 70%, as determined by quench correction series.

F. PROTEIN DETERMINATION

Protein content of whole homogenate, subcellular fractions and 10,000 x g supernatant was determined by the method of Lowry et al. (1951). A 0.2 ml sample of the fraction to be assayed was added to

tubes containing 0.8 ml of 0.5N NaOH. Protein standards at concentrations of 0.5, 1.0 and 1.5 mg/ml were prepared using bovine plasma albumin (Calbiochem). Reagent blanks containing 0.2 ml of Robinson's media were treated identically as tissue samples. The tubes were stoppered and placed in a hot water bath for 1 hour maintained at 70-75°C. The samples were cooled by immersion in cold tap water. A 0.2 ml aliquot of the boiled sample was added to 0.8 ml of 0.5N NaOH and 5.0 ml of Reagent A[a]. The samples were mixed on a Vortex mixer and allowed to stand for 20 minutes at room temperature. Following this, 0.5 ml of Reagent B[b] was added to each sample, mixed immediately and allowed to stand at room temperature for 40 minutes, until color development was complete. The absorbance at 500 nm was read against the reagent blank on a Beckman DB-Spectrophotometer (Beckman Instruments).

G. EXTRACTION OF NUCLEIC ACIDS

The method of Schneider (1945) for the extraction of nucleic acids and the measurement of DNA was used.

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[a]Reagent A: 2.5 ml of a 2.7% (w/v) potassium tartrate solution and 2.5 ml of a 1.0% (w/v) copper sulfate added to 250 ml of 2.0% (w/v) sodium carbonate solution.

[b]Reagent B: Commercial folin-phenol reagent diluted to 1N with distilled water.

A 0.7 ml aliquot of a 10 percent rat uterine whole homogenate was mixed (Vortex) with 2.5 ml cold 10 percent trichloracetic acid (TCA) in 15 ml Corex glass tube and centrifuged in an IEC Clinical Centrifuge at 1000 RPM for six minutes. The supernatant was decanted and discarded and the precipitate resuspended in 2.5 ml of cold 10 percent TCA. The tubes were centrifuged as above, and the supernatant was decanted and discarded. This procedure is used to remove acid-soluble phosphorus compounds.

The tissue residue was suspended in 1.0 ml of distilled water by Vortex mix for 10 seconds, and 4.0 ml of 95 percent ethyl alcohol and centrifuged. The supernatant was decanted and discarded, and the residue was resuspended in 5.0 ml of 95 percent ETOH. The supernatant was decanted and discarded. These steps are used to remove traces of TCA from the residue.

The tissue residue was boiled 3 times for 3 minutes each with 5.0 ml portions of an alcohol-ether mixture (3:1). A small boiling chip was added to each tube in order to facilitate even boiling. The tubes were centrifuged as before following each boiling and the supernatant was decanted and discarded. This procedure was employed to remove phospholipids.

The tissue residue was suspended in 1.3 ml of distilled water mixed with 1.3 ml cold 10 percent TCA and centrifuged. The supernate was decanted and discarded.

The tissue residue was resuspended in 5.0 ml of 5 percent TCA, and the tubes were heated for 15 minutes in a water bath at 90°C. The tubes were centrifuged and the supernatant was decanted and saved.

The tissue residue was resuspended in 2.5 ml of 5 percent TCA and centrifuged. The TCA extracts were combined (7.5 ml) to form the nucleic acid extract.

H. ESTIMATION OF DNA

One ml of the nucleic acid extract was mixed with 2.0 ml of diphenylamine reagent[a] and heated in a boiling water bath for 10 minutes at 90°C. The intensity of blue color was read at 600 nm.

DNA standard solutions were prepared in concentrations ranging from 20 µg/ml to 200 µg/ml by dissolving 10 mg of highly polymerized DNA in 25 ml of 5 percent TCA, and diluting to the desired concentrations. A reagent blank of 5 percent TCA was used.

I. ESTIMATION OF RNA

The method of Ceriotti (1955) was used for the measurement of RNA. Five ml of the nucleic extract was added to 5.0 ml of freshly prepared orcinol reagent[b] in 15 ml screw-cap tubes. The samples were

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[a]Diphenylamine Reagent: 1 gm of diphenylamine which is recrystallized in boiling hexane is dissolved in 100 ml of glacial acetic acid (analytical reagent) and 2.75 ml of reagent concentrated sulfuric acid is added.

[b]Orcinol Reagent: 200 mg of purified orcinol is dissolved in concentrated HCL; add 10 ml of $CuCL_2$ reagent (0.004M $CuCL_2$ in concentrated HCL: 68.2 mg $CuCL_2$/100 ml HCL) and make up to 100 ml with concentrated HCL.

mixed thoroughly and the tubes placed in a boiling water bath for 40 minutes. After cooling with running water, the color was extracted into 5.0 ml of isoamyl alcohol with shaking (Buchler Instruments, New Jersey). The tubes were centrifuged and a 3.0 ml aliquot of the isoamyl alcohol layer, containing the chromophor, was pipetted into an additional series of tubes. The absorbance at 675 nm was determined. RNA standard solutions were prepared by dissolving RNA in 5 percent TCA over low heat. The isoamyl alcohol layer taken from the tube containing no RNA was used as the blank.

J. STATISTICAL METHODS

The 2-tailed students "t" test for independent means, calculated on an Olivetti Underwood Programma 101 desk computer, was used to test for differences between means. The formula employed is as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_p\,(1/N_1) + (1/N_2)}$$

where:

$$S_p^{\,2} = \frac{(N_1-1)S_1^{\,2} + (N_2-1)S_2^{\,2}}{N_1 + N_2 - 2}$$

N_1 = Control sample size

N_2 = Treated sample size

$S_1^{\,2}$ = Control sample variance

$S_2^{\,2}$ = Treated sample variance

$\bar{X}_1$ = Control sample mean

$\bar{X}_2$ = Treated sample mean

The degrees of freedom were taken as $N_1 + N_2 - 2$. The level of significance (P) was determined by comparison of "t" with values from standard tables.

IV. RESULTS

The 10,000 x g supernatant fraction of rat uterine tissue was employed as the histone acetylating system throughout the study (Appendix A and B).

Various concentrations of methylglyoxal and phenylglyoxal were preincubated in a standard assay system for 15 minutes. Histone was added and the reaction was allowed to proceed for an additional 30 minutes. Under these conditions, as shown in Figure 1, both phenylglyoxal and methylglyoxal inhibited in vitro histone acetylation in a concentration dependent manner. Drug concentrations of 2×10^{-4}M to 1×10^{-3}M depressed in vitro histone acetylation approximately 25 percent to 75 percent, respectively (Figure 1). The I_{50} for methylglyoxal was 0.5 mM, and 0.4 mM for phenylglyoxal (Table 1).

The in vitro effect of incubating phenylglyoxal with the uterine histone acetylation preparation prior to assay is shown in Table 2. Following a 10 minute incubation period, an aliquot of the preincubation system containing enzyme and drug was introduced into a standard assay system. The reaction was allowed to proceed for 20 minutes. Under these conditions, approximately 80 percent inhibition was achieved at 5×10^{-4}M assay system concentration, below which level no significant degree of enzyme inhibition was noted. The results of a parallel study, designed to determine the effect of phenylglyoxal

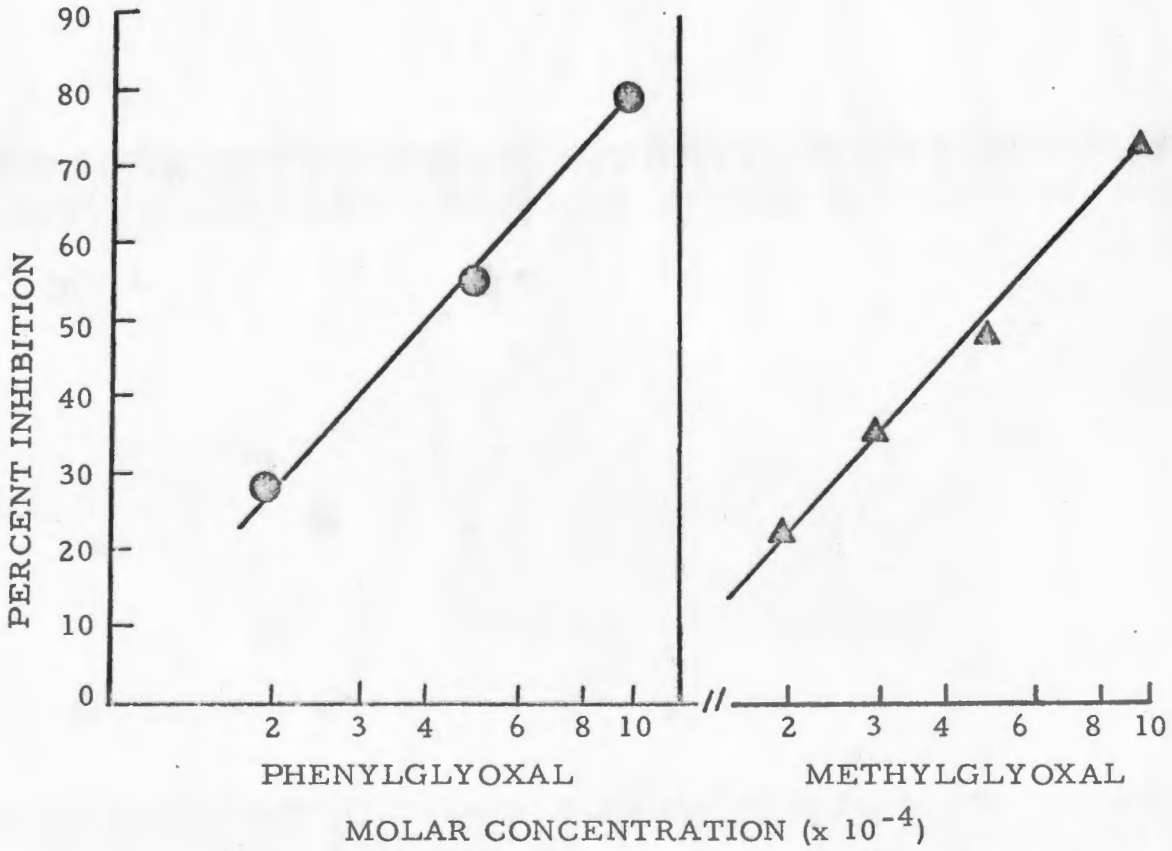

Figure 1: Effect of (a) phenylglyoxal and (b) methylglyoxal on in vitro histone acetylation by a cell-free rat uterine system. Freshly prepared solutions of glyoxals were added at various concentrations and preincubated for 15 minutes at 37°C prior to the addition of histone. The reaction was allowed to proceed for an additional 30 minutes. Each point represents at least three determinations.

Table 1. Inhibition of in vitro histone acetylation by glyoxal derivatives: Index of inhibition (I_{50} value)[a]

Compound	I_{50} Value
Methylglyoxal	0.5mM
Phenylglyoxal	0.4mM

[a]The approximate concentration of drug at which 50 percent inhibition is observed. Various concentrations of inhibitors were preincubated for 15 min at 37°C prior to addition of 1 mg calf thymus histone. The data was plotted and I_{50} values graphically determined.

Table 2. In vitro inhibition of histone acetylation: Effect of phenylglyoxal-enzyme preincubation

Phenylglyoxal Concentration (Final)		Histone Acetylation dpm/mg Histone Mean ± S.E.*	Percent Inhibition
Preincubation System[a]	Assay System[b]		
----	----	22,140 ± 300	control
5.0×10^{-3}M	5.0×10^{-4}M	4,902 ± 591	78
5.0×10^{-4}M	5.0×10^{-5}M	20,117 ± 1521	10
5.0×10^{-5}M	5.0×10^{-6}M	22,317 ± 1011	0

[a] Phenylglyoxal solutions of appropriate concentrations were incubated with 0.25 ml aliquots of 10,000 x g rat uterine supernatant fraction at 37°C for 10 min with shaking.

[b] Following preincubation (a), a 0.1 ml aliquot of the phenylglyoxal-enzyme mixture (equivalent to 0.05 ml supernatant) was added to tubes containing 0.9 ml of standard assay system components, less supernatant. The completed system was then incubated at 37°C, with shaking, for 20 min.

* Mean ± S.E. of triplicate determinations.

incubation with histone are presented in Table 3. In this study, a 5×10^{-4}M phenylglyoxal assay system concentration inhibited in vitro histone acetylation by approximately 30 percent. Inhibition of histone acetylation was not evident at a concentration of 5×10^{-6}M phenylglyoxal.

The effect of glyoxals on uterine weight and total body weight gains in immature rats was explored by administering daily injections of methylglyoxal-bis-guanylhydrazone (Methyl-GAG, 20 mg/kg, intraperitoneally) and glyoxal monohydrate (200 mg/kg, intraperitoneally) for six days. The increase in total body weight over the six day treatment period was also studied. Results of this study are presented in Table 4. Animals receiving glyoxal monohydrate gained significantly less weight ($P < 0.05$) than did the control animals. Uterine wet weight of glyoxal treated animals was approximately 50 percent of vehicle treated controls. This alteration was reflected when the data was expressed as uterine/body weight ratio. This value was employed to account for variation in tissue weight that may be due to body weight differences in animals from any experimental group. No difference ($P > 0.05$) in net body weight gain was observed in animals receiving Methyl-GAG, 20 mg/kg, intraperitoneally, for six days when compared to controls. However, uterine wet weight of Methyl-GAG treated animals was depressed ($P < 0.025$), but to an extent less than that noted in the glyoxal treated group. This alteration was also reflected

Table 3. In vitro inhibition of histone acetylation: Effect of phenylglyoxal-histone preincubation

Phenylglyoxal Concentration (Final)		Histone Acetylation dpm/mg Histone Mean ± S.E. *	Percent Inhibition
Preincubation System[a]	Assay System[b]		
----	----	26,553 ± 3666	control
5.0×10^{-3}M	5.0×10^{-4}M	18,303 ± 4378	32
5.0×10^{-4}M	5.0×10^{-5}M	22,700 ± 1255	15
5.0×10^{-5}M	5.0×10^{-6}M	26, 740 ± 1691	0

[a] 0.6 ml histone solution (10 mg/ml) was added to 0.6 ml of phenylglyoxal solution of various concentrations, or 0.6 ml of phenylglyoxal vehicle (phosphate buffer) for control assays and the mixture was incubated with shaking for 10 min at 37°C.

[b] Following preincubation period, a 0.2 ml aliquot of phenylglyoxal-histone mixture (equivalent to 1 mg histone) was added to tubes containing 0.8 ml of standard assay system components, less histone. The complete system was then incubated at 37°C for an additional 20 min.

* Mean ± S.E. of triplicate determinations.

Table 4. Effect of glyoxal derivatives on body weight and uterine wet weight in immature female rats

Treatment[a]	Body Weight[b] Increase (gm) Mean ± S.E. (N)	Uterine Wet Weight (mg) Mean ± S.E. (N)	Uterine/Body Ratio (%) Mean ± S.E. (N)
Control	34.4 ± 3.1 (5)	152 ± 15 (5)	0.140 ± .014 (5)
Glyoxal	21.2 ± 3.6 (4) ($P < .05$)*	68 ± 5 (4) ($P < .001$)*	0.065 ± .004 (4) ($P < .0025$)*
Methyl-GAG	32.0 ± 2.9 (5) ($P > .05$)*	107 ± 8 (5) ($P < .025$)*	0.092 ± .008 (5) ($P < .01$)*

[a]Control: 1 ml/kg, i.p., 0.5M phosphate buffer (pH 7.4) daily for 6 days; Glyoxal: 200 mg/kg, i.p.; and Methylglyoxal-bis-guanylhydrazone (Methyl-GAG), 200 mg/kg, i.p., daily for 6 days.

[b]Mean body weight gain during 6 day experimental period.

*Determined by Student "t" test.

in the uterine/body weight ratio, indicating that the alteration was not due to inherent tissue weight differences attributable to variations in total body weight alone.

In order to determine the effect of prior glyoxal treatment in animals receiving estradiol alone, several groups of rats were given daily injections of glyoxal, 200 mg/kg, intraperitoneally, or Methyl-GAG, 20 mg/kg, intraperitoneally, for six days. In addition, estradiol 12 μg/rat was administered subcutaneously every 12 hours for 60 hours beginning day three of treatment. The comparison of effect of estradiol alone or in animals pretreated with glyoxals is presented in Table 5. Uterine/body weight ratios of animals receiving either glyoxal or Methyl-GAG prior to estradiol administration were significantly lower ($P < 0.005$) than were comparable values obtained from animals receiving estradiol alone. No significant differences were observed in the liver/body weight ratio of animals receiving estradiol alone or in combination with either glyoxal or Methyl-GAG.

The early effect of estradiol administration on histone acetylation activity in immature rat uterus was explored by sacrificing groups of hormone treated animals at 0, 5, 10 and 15 minutes following injection of estradiol, 12 μg/rat, subcutaneously. The soluble uterine histone acetylation fraction was subsequently prepared (as described previously) and the enzyme activity determined (Figure 2). In addition, a second series of animals received phenylglyoxal, 250 mg/kg, intraperitoneally, 1 hour prior to estradiol administration, in order to determine the

Table 5. Effect of combined glyoxal and Methyl-GAG with estradiol administration on uterus/body ratio and liver/body ratio in immature female rats: Comparison between glyoxal pretreated and estradiol treated animals

Treatment[a]	Uterus/Body Ratio (%) Mean ± S.E. (N)	Liver/Body Ratio (%) Mean ± S.E. (N)
Estradiol-17-β	0.197 ± 0.006 (5)	5.51 ± 0.21 (5)
Glyoxal plus Estradiol	0.104 ± 0.006 (5) ($P < .005$)*	6.17 ± 0.29 (5) ($P > .05$)*
Methyl-GAG plus Estradiol	0.147 ± 0.010 (5) ($P < .005$)*	5.09 ± 0.35 (5) ($P > .05$)*

[a]Estradiol-17-β: 10 μg/rat, sub.Q., every 12 hrs for 60 hrs beginning day 3 of experimental period; Glyoxal monohydrate, 200 mg/kg, i.p. daily for 6 days plus estradiol, 10 μg/rat every 12 hrs for 60 hrs beginning day 3 of experimental period; Methyl-glyoxal-bis-guanylhydrazone (Methyl-GAG), 20 mg/kg, i.p., daily for 6 days, plus estradiol, 12 μg/rat every 12 hrs for 60 hrs beginning day 3 of experimental period.

*Determined by Student "t" test.

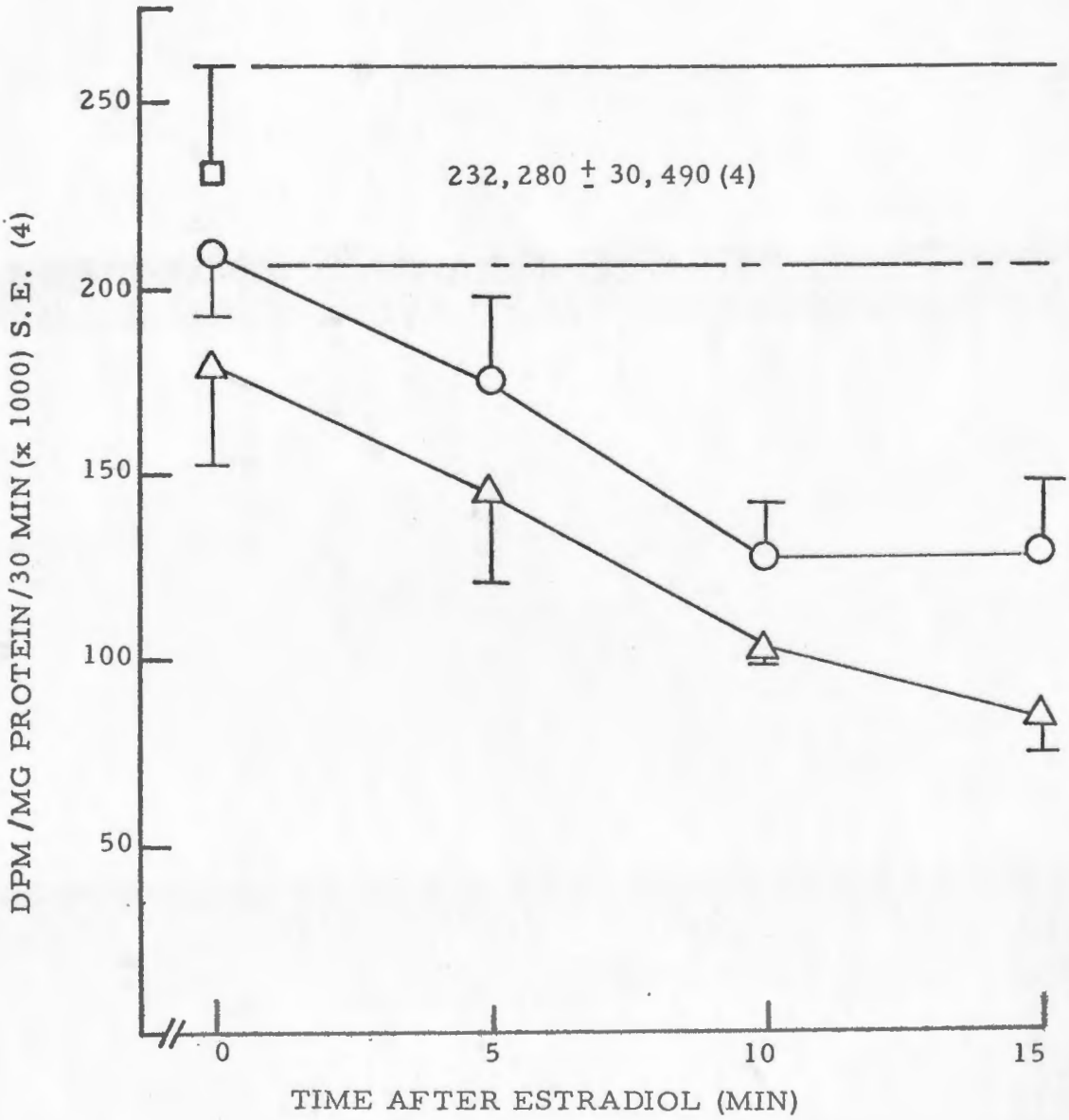

Figure 2: Histone acetylation in soluble uterine fraction from rats treated with estradiol alone and in combination with phenylglyoxal. Rats were administered either 1 ml/kg of 0.05M phosphate buffer vehicle (□); estradiol, 12 μg/rat, sub-Q, (○—○); or phenylglyoxal, 250 mk/kg, i.p., for 1 hour followed by estradiol (△—△). Rats were killed at the time intervals indicated and soluble uterine enzyme activity measured.

effect of phenylglyoxal pretreatment on any early hormone-mediated alterations in uterine histone acetylation. Enzyme activity was significantly depressed ($P < .05$) when measured 10 and 15 minutes following estradiol treatment. A further decrease in enzyme activity was noted in animals pretreated with phenylglyoxal one hour prior to estradiol administration. The differences seen with estradiol treatment alone were significantly different ($P < 0.05$) from phenylglyoxal pretreated animals given estradiol for 15 minutes.

The in vivo effect of estradiol on uterine wet weight and histone acetylation in immature female rats was monitored at 1, 2, 4, 8, 16, and 24 hours following hormone treatment. In addition, a parallel study was performed using phenylglyoxal, 250 mg/kg, intraperitoneally, in order to determine the time course of the early phenylglyoxal mediated decrease in uterine histone acetylation noted in the previous study. The alterations in histone acetylation activity following estradiol and phenylglyoxal administration are presented in Figure 3. A very rapid estradiol mediated decrease in the ability of the uterine enzyme preparation to acetylate histone was found to occur following hormone administration. Enzyme activity remained almost completely depressed until 8 hours after estradiol administration, when enzyme activity began to increase during an 8 to 12 hour period. During 16 to 24 hours, the initial effect of the hormone was reversed, so that at 24 hours post estradiol, enzyme activity was increased to 135 percent of control.

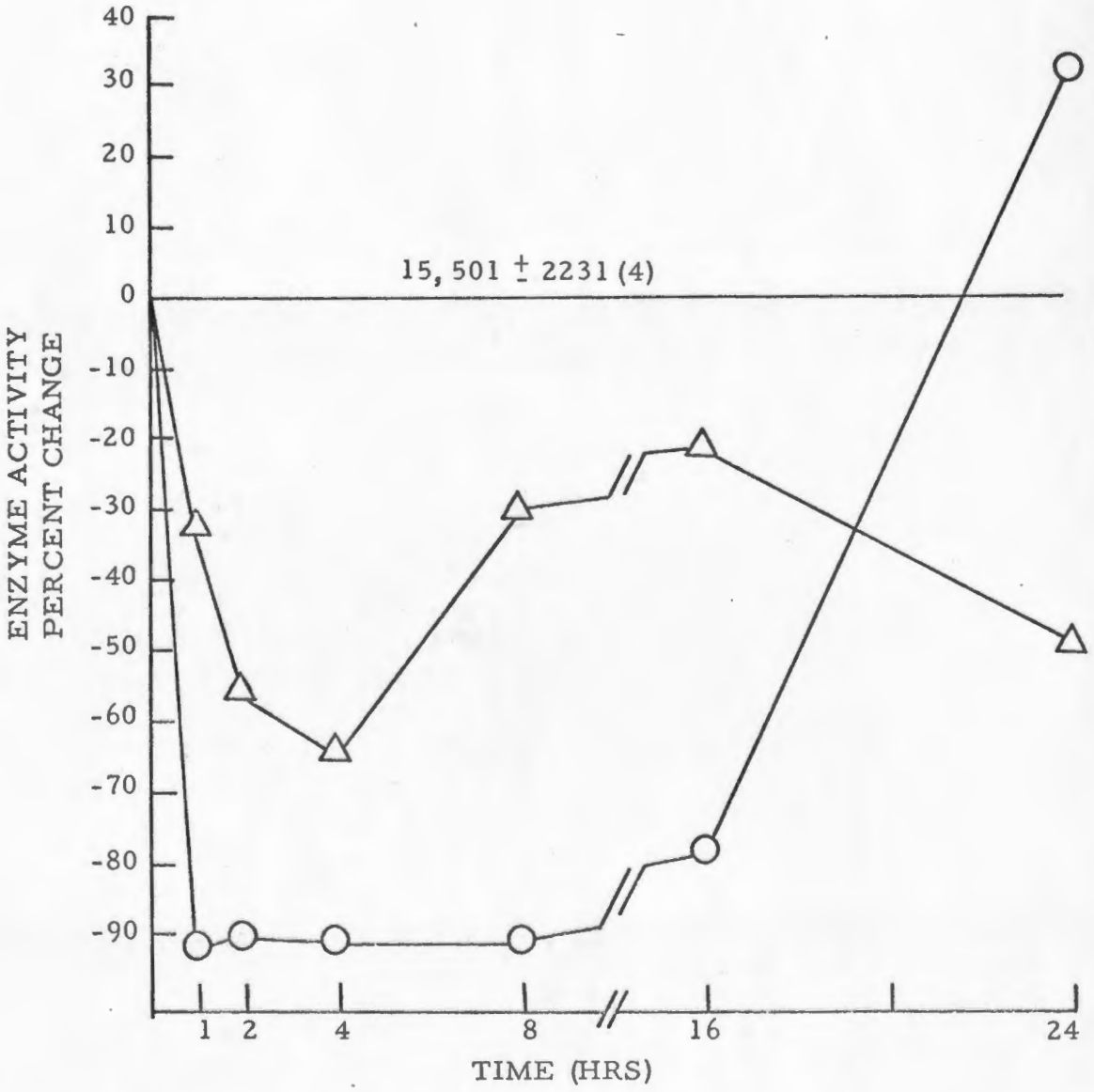

Figure 3: Alteration of histone acetylation by estradiol and phenylglyoxal. Each point represents the mean of at least three values, each obtained from the pooled tissue of two animals. Enzyme activity of control animals is expressed as Dpm/mg protein/30 min. (○—○) estradiol, 10 μg/rat, i. p.; (△—△) phenylglyoxal, 250 mg/kg, i. p.

Enzyme activity response to phenylglyoxal administration closely followed the response seen with estradiol. Uterine enzyme activity decreased rapidly, although not as markedly as noted following estradiol treatment. Reversal of the phenylglyoxal mediated depression in enzyme activity began 4 to 8 hours following drug administration. However, enzyme activity had not returned to control levels 24 hours after phenylglyoxal. A significant ($P < 0.05$) elevation in uterine wet weight was seen two hours after estradiol administration. The initial, rapid elevation was followed by a second but less dramatic one which began at about 6 hours after hormone treatment. At 24 hours, uterine wet weight was elevated to approximately 180 percent of control. Uterine wet weight of phenylglyoxal treated animals did not change significantly ($P > 0.05$) from control values when the parameter was measured over the 24 hour period following drug administration (Figure 4).

Having determined the time course of estradiol and phenylglyoxal activity, it was of interest to determine whether the glyoxals, methylglyoxal and phenylglyoxal, were capable of altering the elevation in uterine histone acetylation and wet weight which had been observed 24 hoursfollowing estradiol treatment. At 20 hours following either estradiol vehicle or hormone treatment, (estradiol, 10 µg/rat, intraperitoneally) groups of animals received either methylglyoxal, phenylglyoxal or a phosphate buffer vehicle. All animals were

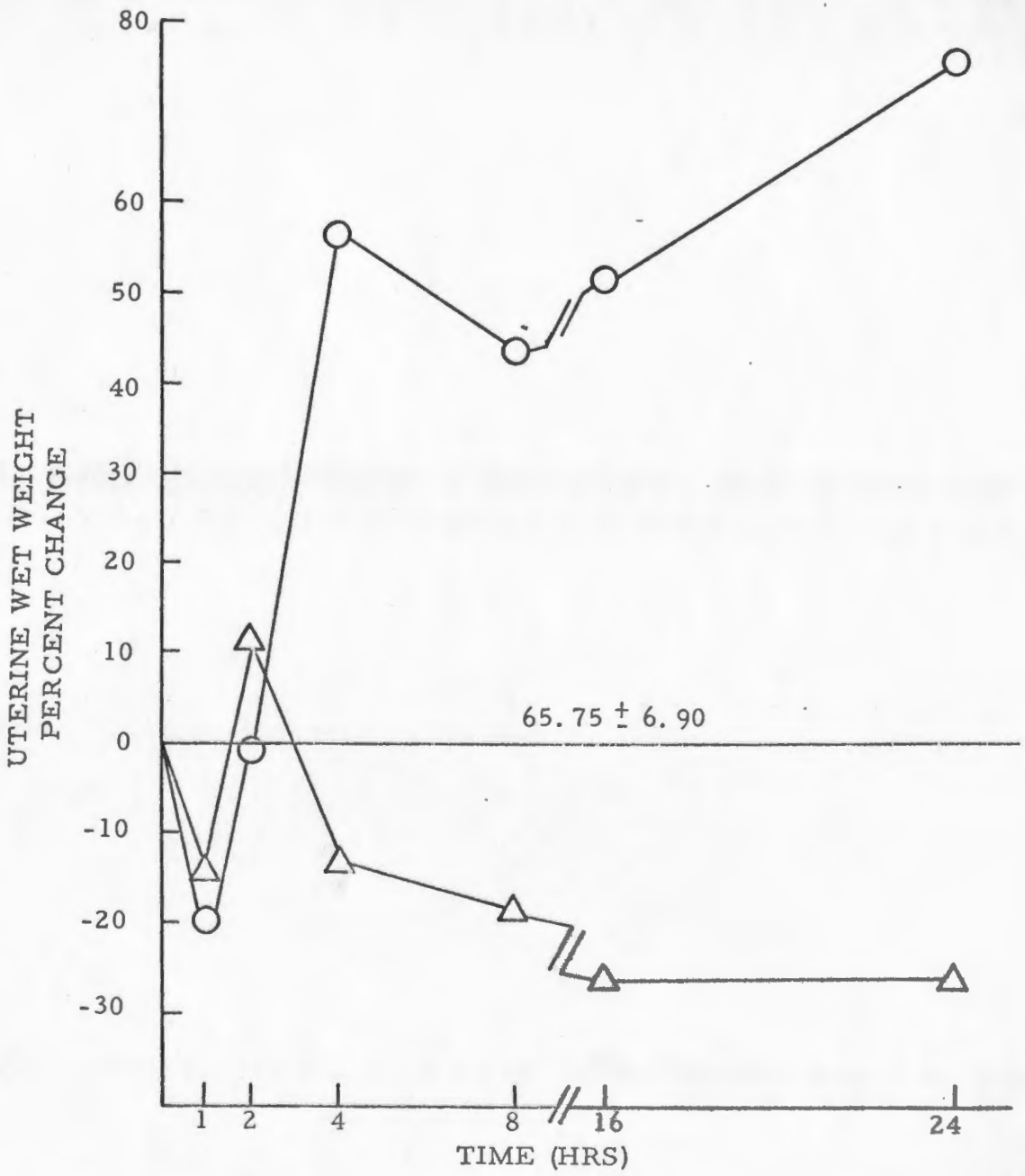

Figure 4: Alteration of uterine wet weight by estradiol and phenylglyoxal. Each point represents the mean of at least six values. Uterine weight of control animals is expressed as mg wet weight. (○—○) estradiol, 10 μg/rat, i.p.; (△—△) phenylglyoxal, 250 mg/kg, i.p.

sacrificed 24 hours following estradiol treatment. Data presented in Table 6 indicate that estradiol-17-β administration causes a rapid increase above controls in uterine wet weight ($P < 0.0025$), 24 hours following hormone administration. Uterine wet weight did not change significantly ($P > 0.05$) from control level when measured 4 hours following methylglyoxal administration at either of the doses used in this study. Weight of uterine tissue obtained from animals treated with estradiol and subsequently with methylglyoxal was elevated above that for vehicle treated controls. Similar results were obtained with phenylglyoxal (Table 7).

The effect of hormone treatment alone and in combination with either methylglyoxal or phenylglyoxal at two dose levels is shown in Tables 6 and 7 respectively. When measured *in vitro* following 24 hour estradiol treatment, enzyme activity was significantly ($P < 0.0005$) elevated above control values. Elevation of uterine enzyme activity 24 hours following estradiol was more pronounced in this study than the increase reported in the estradiol time course study. Variation in both the age of the animals (25 day old *versus* 30 day old rats) and changes in the brand and preparation of the estradiol solution may account for the observed differences.

Methylglyoxal at both dose levels depressed enzyme activity when this parameter was measured 4hours after glyoxal administration

Table 6. Effect of methylglyoxal treatment on uterine weight and histone acetylation in untreated and estradiol pretreated rats

Treatment[a]	Uterine Wet Weight (mg) Mean ± S.E. (N)	Enzyme Activity[b] dpm/mg Protein/30 min Mean ± S.E. (N)
Control	104 ± 12 (6)	12,582 ± 1071 (6)
Estradiol-17-β	178 ± 11 (8) ($P < .0025$)*	26,209 ± 3056 (8) ($P < .0005$)*
Methylglyoxal 125 mg/kg, i.p.	98 ± 11 (7) ($P > .05$)*	8,033 ± 1291 (7) ($P < .025$)*
Methylglyoxal 250 mg/kg, i.p.	104 ± 11 (7) ($P > .05$)*	8,947 ± 877 (7) ($P < .025$)*
Estradiol plus Methylglyoxal 125 mg/kg, i.p.	149 ± 11 (7) ($P < .01$)*	5,022 ± 693 (7) ($P < .0005$)*
Estradiol plus Methylglyoxal 250 mg/kg, i.p.	172 ± 9 (8) ($P < .0005$)*	6,666 ± 717 (8) ($P < .0005$)*

[a] Control: 0.2 ml estradiol vehicle followed by 1 ml/kg 0.05M phosphate buffer (vehicle), i.p. 20 hrs after; Estradiol: 0.2 ml (10 μg)/rat, i.p; Methylglyoxal: 125 mg/kg, i.p. (or 250 mg/kg, i.p.) administered 20 hrs following estradiol vehicle; Estradiol plus Methylglyoxal: estradiol, 10 μg/rat, i.p. followed by methylglyoxal 125 mg/kg, i.p. (or 250 mg/kg, i.p.) 20 hrs after estradiol administration. All animals were sacrificed 24 hrs following estradiol administration (4 hrs following methylglyoxal administration).

[b] Enzyme activity is expressed as the Mean ± S.E. of the amount (dpm) of ^{14}C-acetate incorporated histone formed per mg uterine protein (10,000 x g supernatant) per 30 min.

* Determined by Student "t" test.

Table 7. Effect of phenylglyoxal treatment on uterine weight and histone acetylation in untreated and estradiol pretreated rats

Treatment[a]	Uterine Wet Weight (mg) Mean ± S.E. (N)	Enzyme Activity[b] dpm/mg Protein/30 min Mean ± S.E. (N)
Control	104 ± 12 (6)	12,582 ± 1071 (6)
Estradiol-17-β	178 ± 11 (8) ($P < .0025$)*	26,209 ± 3056 (8) ($P < .0005$)*
Phenylglyoxal 125 mg/kg, i.p.	116 ± 8 (10) ($P > .05$)*	8,215 ± 1153 (10) ($P < .0125$)*
Phenylglyoxal 250 mg/kg, i.p.	109 ± 15 (6) ($P > .05$)*	7,123 ± 1016 (6) ($P < .01$)*
Estradiol plus Phenylglyoxal 250 mg/kg, i.p.	177 ± 13 (10) ($P < .0025$)*	7,638 ± 796 (10) ($P < .0025$)*

[a] Control: 0.2 ml estradiol vehicle followed by 1 ml/kg 0.05M phosphate buffer (vehicle), i.p. 20 hrs after; Estradiol: 0.2 ml (10 µg)/rat, i.p.; Phenylglyoxal: 125 mg/kg, i.p. (or 250 mg/kg, i.p.) administered 20 hrs following estradiol vehicle; Estradiol plus Phenylglyoxal: estradiol, 10 µg/rat, i.p. followed by phenylglyoxal 250 mg/kg, i.p., 20 hrs after estradiol administration. All animals were sacrificed 24 hrs following estradiol administration (4 hrs following phenylglyoxal administration).

[b] Enzyme activity is expressed as the Mean±S.E. of the amount (dpm) of ^{14}C-acetate incorporated histone formed per mg uterine protein (10,000 x g supernatant) per 30 min.

* Determined by Student "t" test.

(Table 6). Similarly, phenylglyoxal treated groups showed lower uterine enzyme activity when measured at the 4 hour interval (Table 7). The glyoxal mediated depression in enzyme activity was also apparent in animals who had received estradiol 20 hours prior to either methylglyoxal or phenylglyoxal (Tables 6 and 7). Elevations in uterine histone acetylation seen 24 hours following estradiol alone were not seen in animals receiving estradiol followed by either glyoxal. Drug induced alterations in uterine histone acetylation are presented in Figure 5 and 6.

A dose response comparison of the effects of the glyoxals on enzyme activity and uterine wet weight is given in Table 8. The depression of histone acetylation by methylglyoxal and phenylglyoxal administered at 125 mg/kg was not significantly different from results obtained at the 250 mg/kg dose level. Similarly, there was no difference between dose levels of either drug on uterine weight of glyoxal treated animals.

The data presented in Table 9 indicate that methylglyoxal and phenylglyoxal administered to animals pretreated with estradiol for 20 hours significantly altered the estradiol mediated elevation of uterine histone acetylation activity seen at 24 hours in animals receiving estradiol alone. Neither methylglyoxal nor phenylglyoxal significantly altered the estradiol mediated increase in uterine weight.

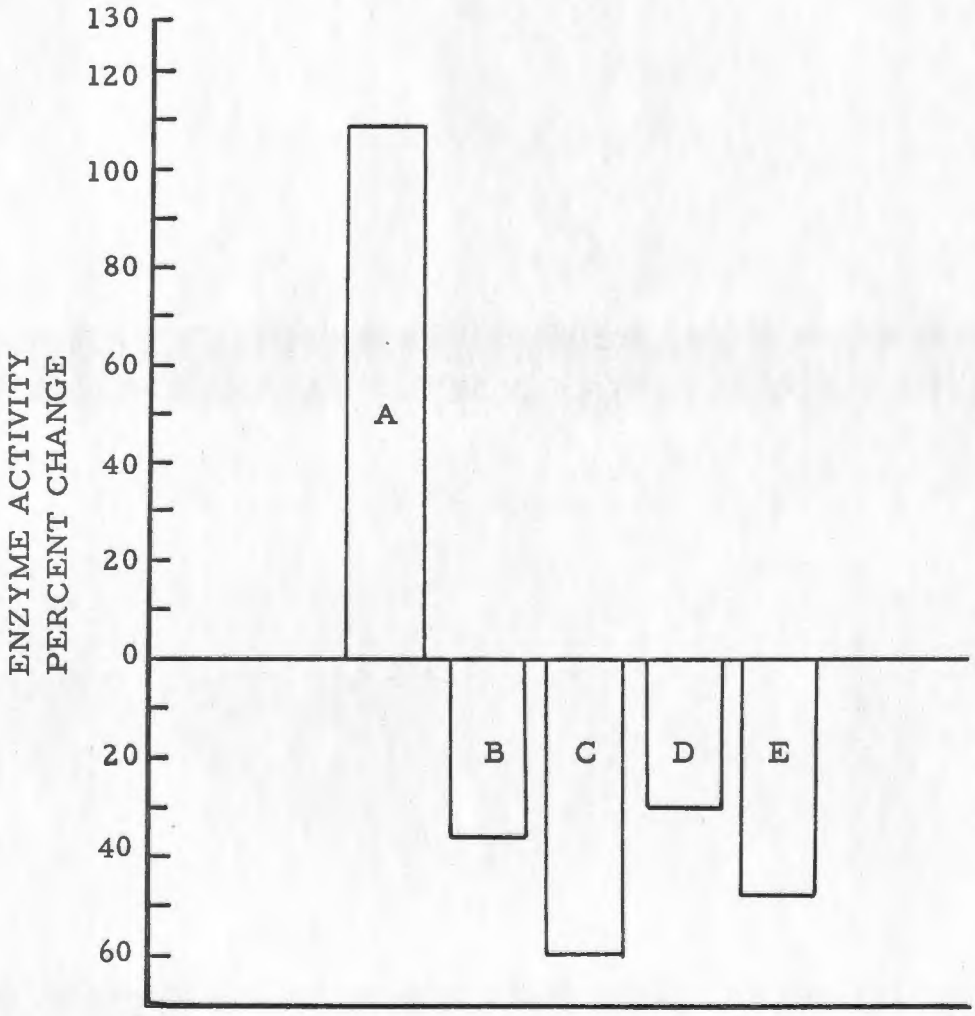

Figure 5: Alteration of histone acetylation in rat uterus by estradiol and methylglyoxal, expressed as percent change from control. Bars represent mean of at least six animals. Drug treated animals received either estradiol and methylglyoxal alone or methylglyoxal 20 hr. post estradiol. All animals were sacrificed 24 hrs. following estradiol administration. (A) estradiol; 10 μg/rat, i.p.; (B) methylglyoxal, 125 mg/kg, i.p.; (C) estradiol plus methylglyoxal, 125 mg/kg; (D) methyglyoxal, 250 mg/kg; (E) estradiol plus methylglyoxal, 250 mg/kg.

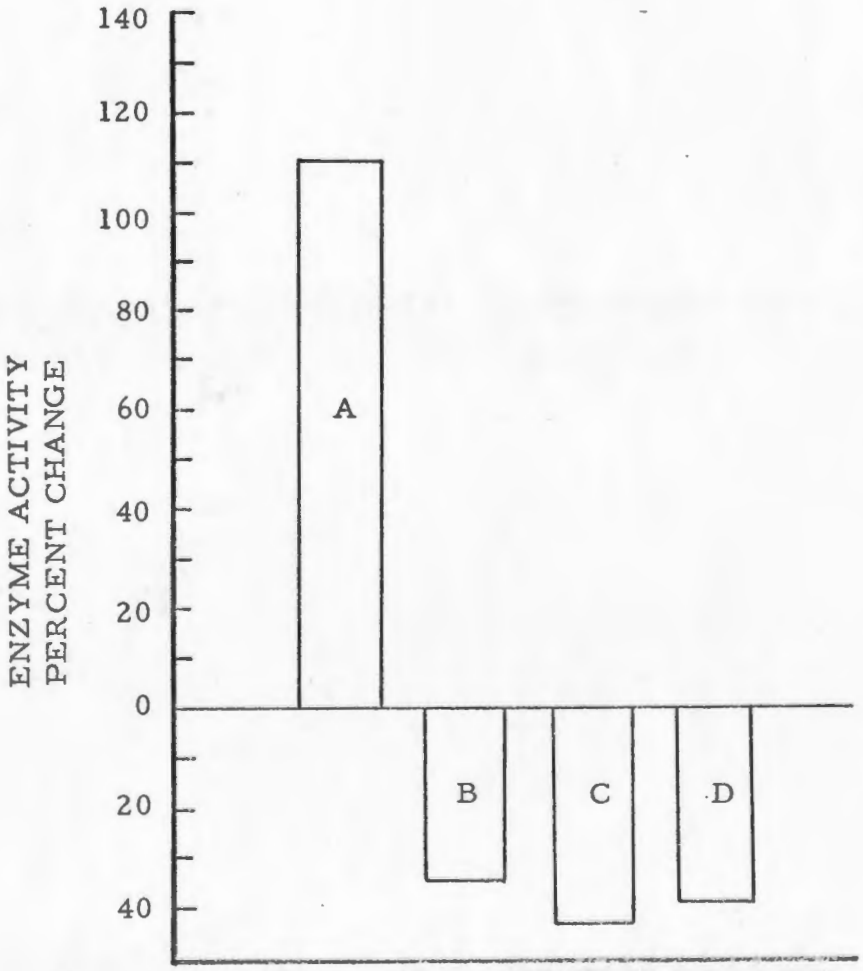

Figure 6: Alteration of histone acetylation in rat uterus by estradiol and phenylglyoxal, expressed as percent change from control. Bars represent the mean of at least six animals. Drug treated animals received either estradiol and phenylglyoxal alone or phenylglyoxal 20 hrs. post estradiol. All animals were sacrificed 24 hrs. following estradiol administration. (A) estradiol, 10 μg/rat, i.p.; (B) phenylglyoxal, 125 mg/kg, i.p.; (C) phenylglyoxal, 250 mg/kg, i.p.; (D) estradiol plus phenylglyoxal, 250 mg/kg.

Table 8. Comparison of alterations in uterine histone acetylation and uterine wet weight at two dose levels of methylglyoxal and phenylglyoxal

Treatment[a]	Uterine Wet Weight (mg) Mean ± S.E. (N)	Enzyme Activity[b] dpm/mg Protein/30 min Mean ± S.E. (N)
Methylglyoxal		
125 mg/kg	98 ± 11	8,033 ± 1291 (7)
250 mg/kg	104 ± 11 (P > .05)*	8,947 ± 871 (7) (P > .05)*
Phenylglyoxal		
125 mg/kg	116 ± 8	8,215 ± 1153 (10)
250 mg/kg	109 ± 15 (P > .05)*	7,123 ± 1016 (6) (P > .05)*

[a] The glyoxals were administered, i.p. 4 hrs prior to sacrifice.

[b] Enzyme activity is expressed as the Mean ± S.E. of the amount (dpm) of ^{14}C-acetate incorporated histone formed per mg uterine protein (10,000 x g supernatant) per 30 min.

* Determined by Student "t" test.

Table 9. Effect of methylglyoxal and phenylglyoxal treatment on the estradiol mediated stimulation of histone acetylation and uterine weight.

Treatment[a]	Uterine Wet Weight (mg) Mean ± S.E. (N)	Enzyme Activity[b] dpm/mg Protein/30 min Mean ± S.E. (N)
Estradiol-17-β	178 ± 11	26,209 ± 3056 (8)
Estradiol plus Methylglyoxal 125 mg/kg, i.p.	149 ± 11 ($P > .05$)*	5,022 ± 639 (7) ($P < .0005$)*
Estradiol plus Methylglyoxal 250 mg/kg, i.p.	172 ± 9 ($P > .05$)*	6,666 ± 717 (8) ($P < .0005$)*
Estradiol plus Phenylglyoxal 250 mg/kg, i.p.	177 ± 13 ($P > .05$)*	7,638 ± 791 (10) ($P < .0005$)*

[a] Estradiol: 0.2 ml (10 μg)/rat, i.p.; Estradiol plus Methylglyoxal: estradiol, 10 μg/rat, i.p. followed by methylglyoxal, 125 mg/kg, i.p. (or 250 mg/kg, i.p.) 20 hrs after estradiol administration; Estradiol plus Phenylglyoxal: estradiol, 10 μg/rat, i.p., followed by phenylglyoxal, 250 mg/kg, i.p. 20 hrs after estradiol. All animals were sacrificed 24 hrs following estradiol administration (4 hrs following administration of either glyoxal).

[b] Enzyme activity is expressed as the Mean ± S.E. of the amount (dpm) of ^{14}C-acetate incorporated histone formed per mg uterine protein (10,000 x g supernatant) per 30 min.

* Determined by Student "t" test.

Table 10 shows the effect of phenylglyoxal, 125 mg/kg, administered 4 hours prior to sacrifice, on uterine weight total DNA, RNA/DNA ratio and protein/DNA ratio. DNA was employed as a baseline in this and parallel studies because, unlike uterine RNA and protein, its concentration remains relatively constant during growth changes and alterations in hormonal environment. When compared to vehicle treated controls, uterine tissue obtained from animals treated for 4 hours with phenylglyoxal did not differ significantly in the various parameters monitored in this study.

Uterine wet weight, total DNA, RNA and protein were measured, and the uterine/body, RNA/DNA and protein/DNA ratios were determined in animals treated with phenylglyoxal alone and in combinations with estradiol. In this study, animals received phenylglyoxal, 125 mg/kg, intraperitoneally, daily for two days. A single injection of estradiol, 10 μg/rat, intraperitoneally, was administered to rats which had received only phosphate buffer vehicle for two days. A second group of rats which had received phenylglyoxal for two days also received a single injection of estradiol. All groups were sacrificed 24 hours following estradiol treatment and subsequent determinations were performed on the excised uterine tissue. The data presented in Table 11 indicate that estradiol given alone elevated uterine wet weight, whereas phenylglyoxal administered for 2 days did not significantly alter this parameter. The uterine weight of

Table 10. Effect of 4 hour phenylglyoxal treatment on wet weight and nucleic acid content in the immature rat uterus

Treatment	Uterine Wet Weight (mg) Mean ± S.E. (N)	Uterine/Body Ratio (%) Mean ± S.E. (N)	Total DNA Pooled Uteri (mg) Mean ± S.E. (N)	RNA / DNA Mean ± S.E. (N)	Protein / DNA Mean ± S.E. (N)
Control[a]	53 ± 5 (12)	0.079 ± .005 (12)	.94 ± .07 (5)	.508	10.7
Phenylglyoxal[b]	54 ± 4 (12) (P > .05)*	0.082 ± .006 (12) (P > .05)*	.83 ± .07 (5) (P > .05)*	.497 (P > .05)*	13.9 (P > .05)*

[a] Control: vehicle, 1 ml/kg, i.p. of 0.05M phosphate buffer (pH 7.4) administered 4 hours prior to sacrifice.

[b] Phenylglyoxal: dissolved in vehicle and administered 4 hours prior to sacrifice, 125 mg/kg, i.p.

* Determined by Student "t" test.

Table 11. Effect of 48 hour phenylglyoxal pretreatment on estradiol-mediated alterations in immature rat uterus

Treatment[a]	Uterine Wet Weight (mg) Mean ± S.E. (N)	Uterus/Body Ratio (%) Mean ± S.E. (N)
Control	62 ± 12 (12)	.082 ± .009 (12)
Estradiol	182 ± 12 (12) ($P < .001$)*	.27 ± .002 (12) ($P < .001$)*
Phenylglyoxal plus estradiol	98 ± 7 (10) ($P < .005$)*	.17 ± .01 (10) ($P < .001$)*
Phenylglyoxal	51 ± 4 (10) ($P > .05$)*	.092 ± .004 (10) ($P > .05$)*

[a] Control: vehicle, 1 ml/kg, i.p. of 0.05M phosphate buffer (pH 7.4) administered once daily for 2 days; Estradiol-17-β; 10 µg/rat, i.p. administered 24 hrs prior to sacrifice; Phenylglyoxal plus Estradiol: 125 mg/kg, i.p. administered daily for 2 days, plus estradiol, 10 µg/rat, single injection on day 2 of treatment. Animals were sacrificed 24 hrs following estradiol; Phenylglyoxal: 125 mg/kg, i.p. administered daily for 2 days. Animals were sacrificed 24 hrs following last injection

*Determined by Student "t" test.

animals pretreated with phenylglyoxal for two days, and then given estradiol was significantly elevated above vehicle treated controls. These alterations were also reflected in the uterine/body weight ratio. As noted in Table 12, total DNA, RNA/DNA ratio, and protein/DNA ratio were significantly elevated in uterine tissue obtained from estradiol treated animals. Measurement of these parameters in uterine tissue of phenylglyoxal treated animals indicate that total DNA was not altered, whereas both the RNA/DNA ratio and protein/DNA ratio were significantly ($P < 0.005$) decreased as compared to vehicle treated controls. The uteri of animals pretreated with phenylglyoxal and then given estradiol contained the same total amount of DNA as was found in vehicle treated controls. However, both the RNA/DNA and protein/DNA ratios were elevated above control values. In Table 13 a comparison is made between the effects of estradiol on the immature rat uterus and the action of the hormone when given to animals who had received phenylglyoxal for 2 days prior to estradiol administration. Uterine wet weight in phenylglyoxal pretreated animals was approximately 50 percent that of estradiol treated animals. Total uterine DNA and the protein/DNA ratio were significantly ($P < 0.05$) decreased when compared to the estradiol treated group. The uterine RNA/DNA ratio of estradiol treated animals did not differ from values obtained from the uterus of phenylglyoxal pretreated animals receiving the hormone.

Table 12. Effect of 48 hour phenylglyoxal pretreatment on estradiol-mediated alterations in immature rat uterus

Treatment[a]	DNA (mg)[b] Pooled Uteri Mean ± S.E. (N)	RNA/DNA Mean ± S.E. (N)	Protein/DNA Mean ± S.E. (N)
Control	0.86 ± .06 (5)	0.600 ± .016 (5)	11.21 ± 0.65 (5)
Estradiol	1.21 ± .09 (6) (P < .02)*	0.825 ± .039 (6) (P < .001)*	18.30 ± 1.18 (6) (P < .001)*
Phenylglyoxal plus estradiol	0.83 ± .12 (5) (P > .05)*	0.831 ± .020 (5) (P < .001)*	13.00 ± 0.31 (5) (P < .05)*
Phenylglyoxal	0.77 ± .06 (5) (P > .05)*	0.490 ± .020 (5) (P < .005)*	7.78 ± 0.30 (5) (P < .005)*

[a] Control: vehicle, 1 mg/kg, i.p of 0.05M phosphate buffer (pH 7.4) administered once daily for 2 days; Estradiol-17- ; 10 ug/rat, i.p. administered 24 hrs prior to sacrifice; Phenylglyoxal plus Estradiol: phenylglyoxal, 125 mg/kg, i.p. administered daily for 2 days, plus estradiol, 10 ug/rat, single injection on day 2 of treatment. Animals were sacrificed 24 hrs following estradiol; Phenylglyoxal: 125 mg/kg, i.p. administered once daily for 2 days. Animals were sacrificed 24 hrs following last injection.

[b] Represents Mean S.E. of total DNA content of samples containing two pooled uteri.

* Determined by Student "t" test.

Table 13. Effect of 48 hour phenylglyoxal pretreatment on estradiol-mediated alterations in the immature rat uterus: Comparison between hormone treatment alone and in combination with phenylglyoxal.

Treatment[a]	Uterine Wet Weight (mg) Mean ± S.E. (N)	DNA[b] Pooled Uteri Mean ± S.E. (N)	RNA / DNA Mean ± S.E. (N)	Protein / DNA Mean ± S.E. (N)
Estradiol	182 ± 12 (12)	1.2 ± .09 (6)	.825 ± .039	18.3 ± 1.2
Estradiol plus Phenylglyoxal	98 ± 7 (10) ($P < .001$)*	.83 ± .12 (5) ($P < .05$)*	.831 ± .02 ($P > .05$)*	13.0 ± .31 ($P < .005$)*

[a]Estradiol-17-β: 10 ug/rat, i.p. administered 24 hrs prior to sacrifice; Phenylglyoxal: 125 mg/kg, i.p. administered daily for 2 days plus estradiol, 10 μg/rat, i.p., single injection on day 2 of treatment. All animals were sacrificed 24 hrs following estradiol.

[b]Represents Mean ± S.E. of total DNA content of samples containing two pooled uteri.

*Determined by Student "t" test.

V. DISCUSSION

The in vitro studies of the effect of various ketoaldehydes on uterine histone acetylation suggest a dose dependent inhibitory action (Figure 1). The nature of the I_{50} study (Table 1) makes it difficult to relate the values for glyoxal concentrations obtained in this study to those found to be effective in other laboratories. The I_{50} value is dependent upon the protein content used in the system being analyzed, in this case, both enzymatic and other cell protein; therefore, the I_{50} values reported in our study will not necessarily correlate with similar studies that used a different protein content in their assay system. It is of interest to note, however, that the various glyoxal derivatives employed in several reported studies (Gregg, 1968; Klamerth, 1968; Szent-Gyorgyi, 1968) were active at concentrations similar to the I_{50} values reported in our studies. Scaife (1969), using mM concentrations, obtained methylglyoxal and kethoxal mediated in vitro inhibition of human kidney cell DNA, RNA, and protein synthesis. Similar concentrations of glyoxal (0.5mM) are capable of blocking DNA replication and protein synthesis without affecting respiration in human fibroblast cell cultures (Klamerth, 1968). In addition, a sharp concentration effect, evident in the dose response study was reported to occur with methylglyoxal when the drug was tested for mitotic inhibitory properties (Scaife, 1969). While a direct correlation between these

literature reports and the present study is not warranted, it is noteworthy that several studies involving different biological systems show that glyoxal derivatives are effective at similar concentrations.

Although inhibition of in vitro histone acetylation had been demonstrated by our previous studies, the mechanism(s) involved in these actions was left undetermined. Results obtained from the study of glyoxal mediated inhibition at the incubation level (Tables 2, 3) suggest that the major site of action is the uterine acetylation enzyme system. However, a direct alteration of histone protein leading to a blockade of acetylation must also be considered, since phenylglyoxal-histone preincubation results in a significant decrease in the in vitro acetylation of histone protein. Takahashi (1968) demonstrated a blockade of guanido residues in protein, which may account in part for the decrease in histone acetylation following incubation of histone protein in the presence of phenylglyoxal.

The results of our in vitro studies led to an analysis of the in vivo effects of the glyoxal derivatives. According to present concepts of histone function(s), blockade of histone acetylation in vivo might be expected to alter normal growth patterns in a biological system. The rat uterus, which had been employed during our preliminary studies, was chosen as a model system in which to study glyoxal action, because of the well defined growth pattern initiated by hormonal stimulation. Although an alteration in uterine histone acetylation

following estradiol treatment had not been reported in the literature prior to the initiation of these studies, it was our belief that hormone treatment would affect this parameter and that hormone-induced alterations in the target organ could be blocked to some extent by the glyoxals. Results obtained from our initial in vivo studies indicated that the two glyoxals, Methyl-GAG and glyoxal monohydrate, depressed uterine wet weight (Table 4). In addition, elevation of uterine wet weight normally observed in estradiol treated rats was not apparent in animals receiving estradiol in conjunction with the glyoxals (Table 5). These data suggest a glyoxal mediated blockade of at least one parameter of estrogen response.

Before any further study involving estradiol, glyoxals, and uterine histone acetylation could be attempted, it was necessary to delineate the action of estradiol on acetylation properties of the hormone stimulated uterus, so that we would then be able to evaluate glyoxal mediated alterations of this system more precisely.

An initial effect of estradiol treatment on the rat uterine acetylation system was the depression of histone acetylation in this organ (Figure 2). This action was very rapid and resulted in a depression in enzyme activity measurable as early as 10 minutes following hormone treatment. Our data are not in agreement with Libby (1971), who reported a rapid stimulation of histone acetylation immediately following estradiol treatment, peaking at approximately 15 minutes and

returning to control levels 20 minutes following estradiol treatment. However, our results do agree with Anderson and Gorski (1971), who demonstrated that estradiol caused an early decrease in histone acetylation followed by an increase, beginning at about 18 hours and elevating to 150 percent of control 24 hours after hormone treatment. Kaye and Sheratzky (1969) have previously shown a decrease in the activity of protein (histone) methylase obtained from the centrifuged (105,000 x g) supernatant fraction obtained from rat uterus of estradiol treated animals. The differences in dose schedule, observation times, and assay procedure may account for the lack of agreement of the results of these studies.

Phenylglyoxal, 250 mg/kg, intraperitoneally, decreased enzyme activity when administered one hour prior to estradiol treatment (Figure 2). The mean enzyme activity for phenylglyoxal pretreated groups receiving estradiol tended to be lower than the mean values of estradiol treated groups at 0, 5, and 10 minutes following hormone treatment. Although not statistically significant ($P > .05$), these values suggest that a longer pretreatment period might result in a greater difference between these groups.

Phenylglyoxal pretreatment, followed by estradiol, significantly lowered ($P < .05$) histone acetylation activity as compared both to control groups and to estradiol treated groups, when measured 15 minutes following estradiol treatment. The apparent early inhibitory

effect upon uterine histone acetylation by estradiol was unexpected in that stimulation of hormonally excitable systems generally results in an early augmentation of histone acetylation (Allfrey, 1966; Pogo et al., 1966; Pogo and Allfrey, 1968).

Data presented in Figure 3 indicate that one of the initial effects mediated by estradiol is a virtually complete inhibition of uterine histone acetylation. The time course of the latter reversal of the initial inhibition and subsequent stimulation of histone acetylation to levels above control may be pertinent when estrogen mediated alterations in uterine RNA and protein content are considered. In the studies of Oliver and Kellis (1970), RNA (µg/mg dry weight) in the immature rat uterus remained relatively constant up to 10 hours after treatment with 5 µg of estradiol. Elevation of uterine RNA levels reportedly occurred between 10 and 12 hours following estradiol, whereas uterine DNA (µg/mg dry weight) decreased during the same interval. Administration of estradiol to ovariectomized rats resulted in a measurable elevation in the RNA/DNA ratio, commencing approximately 8 hours and continuing up to 24 hours after hormone administration. Results of the present study suggest that the estradiol mediated elevation in uterine histone acetylation occurred at a time when uterine RNA levels were increasing through the action of this hormone. These findings are in accord with other workers who have observed an elevation in histone acetylation preceding or

paralleling rises in RNA and protein in several unrelated systems (Allfrey et al., 1963; Pogo et al., 1966; Pogo et al., 1969).

Measurement of uterine histone acetylation activity following phenylglyoxal treatment (Figure 3) demonstrated the ability of this drug to cause a rapid depression in uterine enzyme activity, with peak depression corresponding to approximately 60 percent inhibition 4 hours following phenylglyoxal administration. Enzyme activity remained depressed up to 24 hours. The effect of both estradiol and phenylglyoxal on uterine histone acetylation and alterations in uterine wet weight followed similar temporal patterns during the first 2 hours of drug activity (Figure 4).

The apparent decrease in uterine wet weight following phenylglyoxal treatment was not significant (Figure 4), although it is assumed from the results presented in Table 1 that more prolonged administration of phenylglyoxal would most likely depress uterine wet weight. The rapid elevation in uterine wet weight following estradiol administration to immature female rats is well characterized, and it is thought to be primarily a hypertrophic response. Water uptake in the uterus of estradiol treated rats reaches a peak 4 to 6 hours after hormone treatment. The initial water inhibition was followed by a second similar response after 12 hours (Mueller, 1971). Uterine wet weight, measured 24 hours after hormone administration to ovariectomized rats, has been shown to increase approximately 190 percent above

untreated controls (Hamilton et al., 1968). In our study, uterine wet weight alterations seen in 25-30 day old female rats following estradiol treatment were similar to those previously reported (Figure 4). The physiological effects characteristic of estradiol were evident throughout the period uterine histone acetylation was monitored. It is difficult at this time to present a completely satisfactory explanation of the biological role this hormone plays in its initial action on histone acetylation in its target organ. Barker (1971) showed that the synthesis of arginine-rich histones in rat uterus following estradiol treatment was elevated immediately. The rate of synthesis decreased toward control levels 6 hours after treatment and then became steadily elevated to a 22-fold increase 48 hours following estradiol. Surprisingly, the amount of arginine-rich histone per uterus decreased dramatically to 44 percent of control levels 1 hour following estradiol administration, remaining depressed until approximately 10 hours after estradiol when it returned to control levels; and then when measured 12 hours following hormone treatment, they became elevated to 138 percent of control values. Estradiol mediated alterations in uterine histone synthesis and content observed in these studies somewhat paralleled the pattern of alterations in histone acetylation reported in our work.

These results may offer an explanation of some of the alterations observed following estradiol administration. Barker (1971) suggests

that hydrolysis of histone protein may account for its disappearance in the uterus immediately following estradiol treatment. Paik and Lee (1970; 1971) have reported an enzyme which specifically hydrolyzes histone protein. Earlier, Goodall (1965) reported an increase in uterine proteolytic activity following estradiol administration. We suggest that, if such an enzyme exists in the uterus, the elevation of its activity by estradiol, either through activation mechanisms or through de novo synthesis, would account for the rapid depression in arginine-rich histone content in the estradiol treated uterus. Control mechanisms involved in the depression of histone content by the hormone might also be responsible for a decrease in the activity of an enzyme involved in the control of histone modification. Any continuation of the normal cellular levels of uterine histone acetylation in the face of rapidly decreasing histone levels following estradiol would not be efficient, whereas a corresponding decrease in enzyme activity to parallel the depleted uterine histone content would be reasonable. As the highly elevated rate of histone synthesis negated the effect of elevated histone degradation, normal histone levels would be reestablished and a "feedback" type of control of hormonal stimulation would be accomplished. A return of uterine histone acetylation capacity following estradiol would be expected to follow the observed rise in histone content, since histone modification would again be required.

Barker's observation that the levels of uterine arginine-rich histones were elevated to above control values 12 hours after estradiol coupled with our findings that such elevation precedes the reversal in the initial depression in histone acetylation, are consistent with this proposed model of estradiol mediated alterations in histone metabolism. We suggest that an alteration in histone metabolism other than by acetylation may be implicated in the initial derepression mechanisms thought to occur in the estradiol stimulated rat uterus.

Our previous study established that estradiol elevates uterine histone acetylation when measured 24 hours following hormone treatment. Furthermore, phenylglyoxal depressed this parameter with peak depression in activity occurring 4 hours following drug administration. By aligning these time intervals, it was possible to measure the effect of glyoxal treatment on the established elevation of uterine histone acetylation 24 hours after hormone treatment. Uterine wet weight was utilized as a secondary measurable parameter to monitor the net physiological action of estradiol and phenylglyoxal. The data obtained from this study suggest that 4 hour treatment with either methylglyoxal or phenylglyoxal is ineffective in itself in altering uterine wet weight significantly (Tables 6, 7). Similarly, the elevations in uterine wet weight in estradiol treated animals receiving phenylglyoxal or methylglyoxal suggest that the glyoxals do not reverse the initial uterine hypertrophy following hormone treatment manifest

at the time of glyoxal administration. These data are consistent with the view that early water imbibition of the uterus, which accounts for most of the elevated uterine wet weight following estradiol, is an extragenomic action of the hormone. Ui and Mueller (1963) have shown that water imbibition of the uterus following estradiol takes place even when uterine RNA synthesis has been blocked by actinomycin-D; their findings support the contention that the process of uterine imbibition is not completely under genomic control. It is therefore conceivable that the depression of uterine histone acetylation activity by methylglyoxal and phenylglyoxal treatment or the apparent reversal of the estradiol mediated elevation in enzyme activity (Tables 6, 7; Figures 5, 6) might not be expected to influence all measurable parameters in the uterus in a parallel manner.

An analysis of uterine tissue obtained from phenylglyoxal treated animals 4 hours after drug administration failed to show any alteration in the parameters monitored in this study (Table 10). Since uterine histone acetylation is depressed by this treatment, it is evident that the depressed enzyme activity precedes any measurable alteration in nucleic acids and protein content, as reflected by the total DNA, RNA/DNA and protein/DNA ratios. Pogo et al. (1966) reported that RNA synthesis in equine polymorphonuclear leucocytes following treatment with phytohemaglutinin in vitro was diminished. Furthermore, preliminary experiments suggest that an inhibition of histone acetylation

in this system may precede the observed decrease in RNA synthesis. Since we have measured levels rather than synthesis of uterine nucleic acids following 4 hours glyoxal treatment, it was not unexpected that little or no alteration in nucleic acid content following short term glyoxal treatment was noted. However, by extending the period of glyoxal treatment from 4 to 48 hours, it was possible to demonstrate a more pronounced effect of phenylglyoxal on the uterus of estradiol treated animals. Although phenylglyoxal does not lower uterine wet weight when given alone, the drug is capable of decreasing the magnitude of uterine weight gain seen after administration of the hormone (Table 11). This action is significant in that it demonstrates the ability of an endogenous metabolite to interfere with the physiological action of a steroid hormone. Alterations of nucleic acid and protein constituents of the estrogen stimulated uterus become apparent when measured in phenylglyoxal pretreated animals receiving the hormone (Table 12). Elevations in total uterine DNA and the protein/DNA ratio following estradiol are not attained in phenylglyoxal pretreated animals (Table 13). Phenylglyoxal apparently does not affect the estrogen mediated elevation of the DNA/RNA ratio. These data are in general agreement with other studies concerned with the action of the glyoxals in various biological systems. In studies using human kidney cells, Scaife (1969) found that kethoxal and methylglyoxal were potent inhibitors of protein synthesis in this system; DNA

synthesis was partially blocked, whereas RNA synthesis was only slightly influenced. A similar pattern of glyoxal mediated alterations in DNA, RNA and protein synthesis has been described by Szent-Gyorgyi (1967). Klamerth (1968) has also reported that the principal effect of glyoxal (0.5mM) on human cells grown in culture is a depression in both DNA and protein synthesis, leading him to believe that DNA replication rather than transcription is being blocked by the glyoxals.

In light of the results presented here and in related studies, the possible consequences of glyoxal mediated inhibition of the acetylation of histones involved in the control of DNA replication warrant consideration if a mechanism of action is to be determined for the known biological activity of the ketoaldehydes. If the ketoaldehydes are important agents in the regulation of histone acetylation, this suggests that they may be therapeutically useful in inducing a termination of cellular proliferation.

VI. SUMMARY AND CONCLUSIONS

The ketoaldehydes, phenylglyoxal and methylglyoxal, inhibit the *in vitro* acetylation of histones by a cell-free soluble fraction of rat uterus. The degree of enzyme inhibition is dose related. As determined by the reported I_{50} value for each drug, methylglyoxal and phenylglyoxal are equally effective in inhibiting histone acetylation.

In vitro inhibition of uterine histone acetylation by phenylglyoxal is thought to be accomplished primarily by inhibition of the uterine enzyme system. However, the formation of a glyoxal-histone complex which is a product less capable of undergoing acetylation is also suggested by our data. The importance of these actions *in vivo* cannot be assessed at this time.

Immature female rats treated for six days with glyoxal monohydrate gain less weight than vehicle treated controls, whereas Methyl-GAG had no effect on this parameter. The decrease in uterine wet weight following glyoxal treatment was not as evidence in Methyl-GAG treated rats. Estradiol mediated elevations in uterine/body ratios are blocked or reversed by prior treatment with Methyl-GAG and glyoxal monohydrate, respectively. Uterine/body weight ratios but not liver/body weight ratios were altered when measured in animals receiving the glyoxals and estradiol.

The hormonally stimulated immature rat uterus was developed as a model system in which to measure estradiol and ketoaldehyde

induced alterations in uterine histone acetylation. Various parameters were monitored in order to delineate more clearly the actions of both the glyoxals and estradiol on the physiology and biochemistry of the system under study. The temporal relationships of the alterations induced by these agents may offer an alternate explanation for the biological action of both the hormone and the proposed inhibitor.

A rapid depression in uterine histone acetylation occurred following estradiol administration to immature rats. The initial hormonal action on uterine histone acetylation was reversed over the initial 24 hours, leading to an elevation above control levels. The depression in uterine histone acetylation occurred during the period in which hormonally induced elevations in uterine wet weight were observed. These findings suggest that an elevation in histone acetylation may not be an initial event in the general stimulation of the immature uterus by estradiol.

Four hour treatment with methylglyoxal and phenylglyoxal reversed the estradiol mediated elevation in uterine histone acetylation, without affecting uterine wet weight. The phenylglyoxal mediated inhibition of uterine histone acetylation preceded any alteration in uterine wet weight, total DNA, RNA/DNA or protein/DNA ratio.

When the duration of phenylglyoxal treatment was extended to 48 hours in order to measure the effectiveness of the drug in altering

the actions of estradiol on various parameters of the model system under study, depressions in uterine RNA/DNA and protein/DNA ratios occurred. Estradiol-mediated elevations in uterine wet weight, total DNA and the protein/DNA ratio were partially blocked by this treatment. The drug was ineffective in blocking the elevation in RNA/DNA ratio produced by estradiol. The alteration of normal physiological activity in the immature rat uterus by phenylglyoxal became most apparent when this action was monitored in a hormonally activated system.

Although no direct correlation between the initially depressed rate of histone acetylation preceding the decrease of uterine nucleic acid levels can be made, we suggest that blockade of the normal pattern of histone modification following hormone administration may lead to parallel depressions in levels of key intermediates necessary for the complete accomplishment of hormonal action.

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VII. VITA

Robert Louis Procaccini was born on February 9, 1942 to Mr. & Mrs. Domenic Procaccini in Providence, Rhode Island. Mr. Procaccini completed his elementary education requirements in Providence, Rhode Island. In 1959, Mr. Procaccini graduated from LaSalle Academy in Providence and entered the University of Rhode Island where he received the Bachelor of Science Degree in Pharmacy in June, 1964. Requirements for license as a Registered Professional Pharmacist of the State of Rhode Island were completed in July, 1964. Mr. Procaccini received the Master of Science Degree in Pharmacology in June, 1969, and the Doctor of Philosophy Degree in Pharmaceutical Science in June, 1972. He is a member of Theta Delta Chi Fraternity and the Rho Chi and Phi Sigma Societies.

Mr. Procaccini is married to the former Ruth Ann Brooks of Tiverton, Rhode Island.

APPENDIX

The data presented in this appendix is complementary in nature and serves to more clearly characterize the uterine histone acetylation system employed in this study.

Significant histone acetylation activity was observed in the mitochondrial, microsomal and supernatant (100,000 x g) fraction of rat uterus obtained from immature (25 day old) rats. Specific activities of 16,255, 31,219 and 157,563 DPM/mg protein were observed for these fractions, respectively (Appendix A). These data, expressed as percent total activity *versus* percent total protein is represented in Appendix B. Enzyme activity was highest in the soluble (supernatant) rat uterus fraction.

The effect of temperature on *in vitro* histone acetylation is represented in Appendix C. Enzyme activity remains constant from 30°C to 38°C followed by an abrupt elevation at 40°C assay temperature. Enzyme activity declined sharply as the temperature exceeded 40°C and approached 60°C. The Q_{10} (37-45°C) value of the acetylating enzyme system is 1.77.

The elevation in histone acetylation activity at 40°C was used as a basis for studying reversibility of this activity. Enzyme preparations preincubated at an elevated temperature (40°C) retained their elevated acetylation activity when assayed at the standard incubation temperature of 37°C (Appendix D). Enzyme preparations containing equal

aliquots of enzyme preincubated at 37°C and at 40°C demonstrated activity slightly less than additive

APPENDIX A

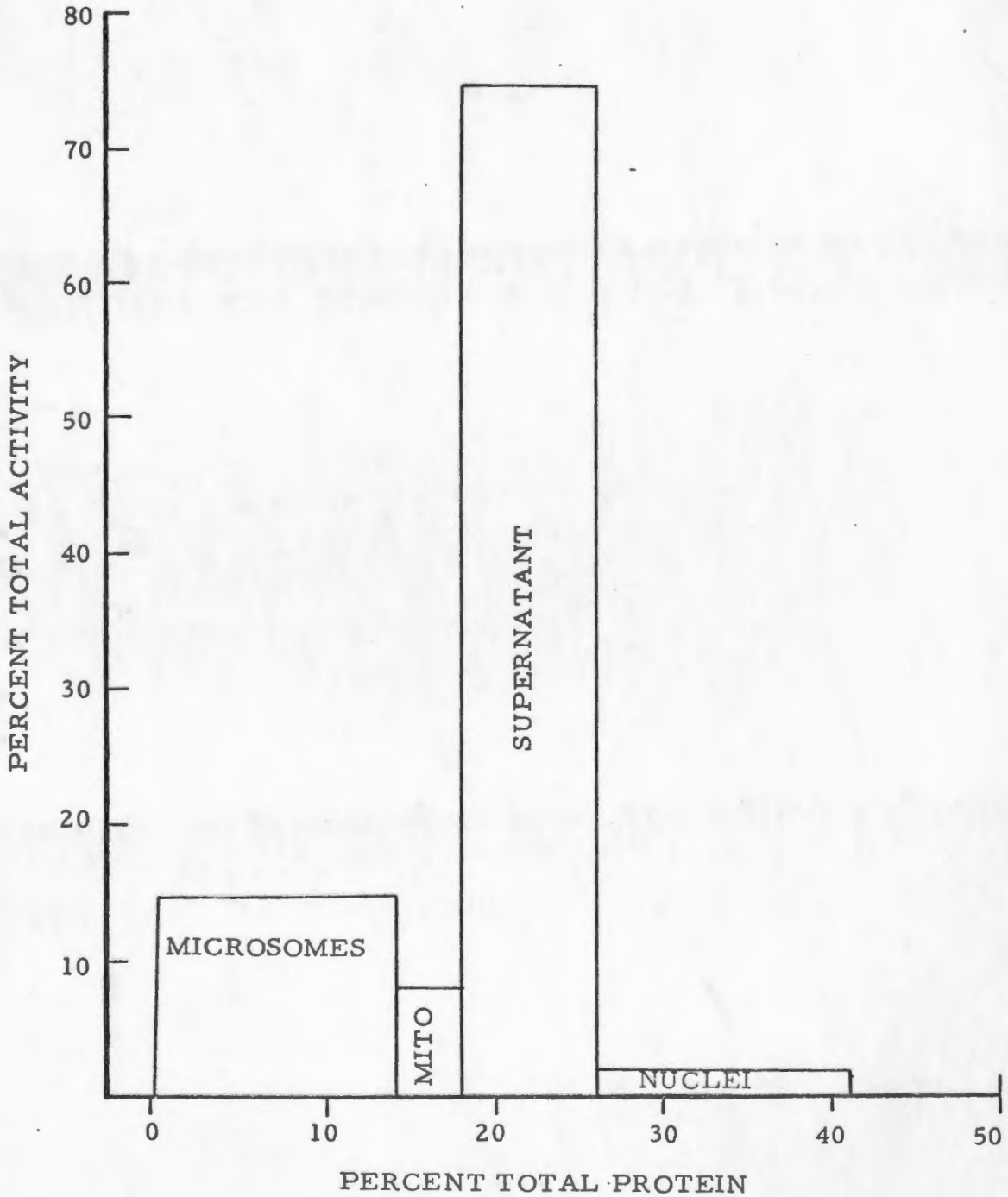
Subcellular Localization and Specific Activity of Histone Acetylation In Immature Rat Uterus

Fraction[a]	Specific Activity (dpm/mg protein)	Percent of Total Activity
Nuclei	4,610	2.2
Mitochondria	16,255	7.8
Microsomes	31,219	14.8
Supernatant (105,000 x g)	157,563	75.2

[a]A weighed sample of rat uterus was homogenized in 0.25M sucrose and centrifuged at 780 x g for 15 minutes, and 105,000 x g for 60 minutes to obtain nuclear, mitochondrial, and microsomal pellets, respectively. Pellets were washed in 0.25M sucrose and resuspended to volume in Robinson's media (Robinson, 1949). A 0.05 ml aliquot of each fraction was used as enzyme source for assay of histone acetylation.

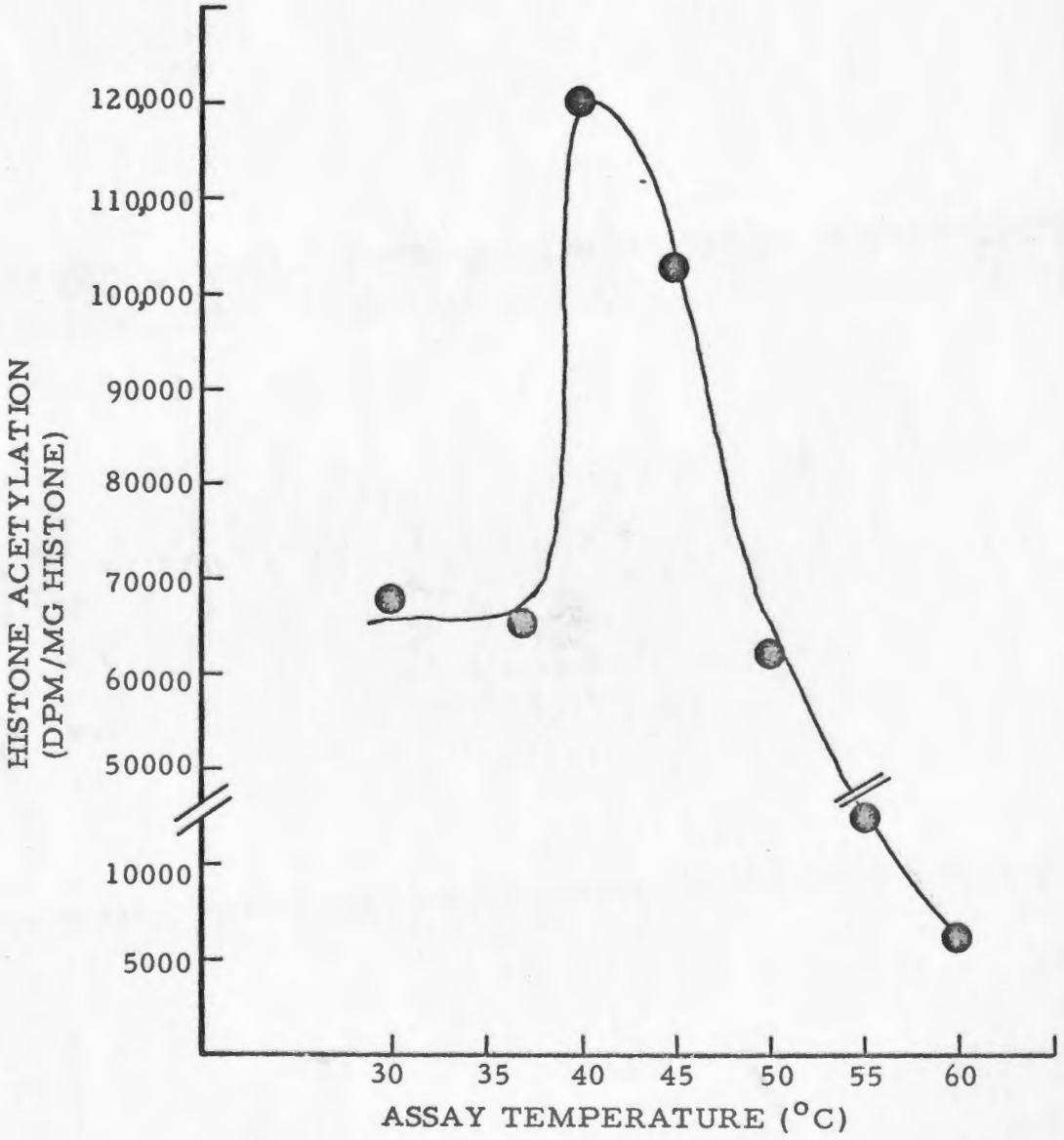
APPENDIX B

Subcellular Localization and Specific Activity of Histone Acetylation In Immature Rat Uterus


80
70
60
50
40
30
20
10
PERCENT TOTAL ACTIVITY
MICROSOMES
MITO
SUPERNATANT
NUCLEI
0
10
20
30
40
50
PERCENT TOTAL PROTEIN

APPENDIX C

Effect of Temperature on *in vitro* Histone Acetylation


HISTONE ACETYLATION
(DPM/MG HISTONE)
120,000
110,000
100,000
90000
80000
70000
60000
50000
10000
5000
30
35
40
45
50
55
60
ASSAY TEMPERATURE (°C)

APPENDIX D

In vitro Thermal Activation of Histone Acetylation Properties of Rat Uterine Supernatant Fraction

Series	Preincubation Temperature	Assay Temperature	Histone Acetylation (dpm/mg Histone) Mean ± S.E.*
I[a]	37°C	37°C	13,793 ± 1885
II[b]	40°C	37°C	26,313 ± 2680
III[c]	37°C 40°C	37°C	18,220 ± 1115

[a]Standard assay system using 10,000 x g rat uterine fraction (0.1 ml) preincubated for 20 min at 37°C, cooled on ice and added to complete assay system for incubation at 37°C for an additional 15 min. Prior to incubation, uterine fraction was diluted (1:1) with Robinson's media.

[b]Standard assay system using 10,000 x g rat uterine fraction (0.1 ml) preincubated for 20 min at 40°C, cooled on ice and added to complete assay system for incubation at 37°C for an additional 15 min. Uterine fraction was diluted (1:1) as above.

[c]Standard assay system using combined supernatant fraction prepared by mixing 0.1 ml aliquots of undiluted Series I and II treated supernatant with 0.2 ml Robinson's media. At time of assay, 0.1 ml of final mixture was added to complete assay system for incubation at 37°C for 15 min.

*Mean ± S.E. (for three determinations).