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HORMONAL AND DIETARY REGULATION OF ENZYME

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by

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ABSTRACT

HORMONAL AND DIETARY REGULATION OF ENZYME INDUCTION IN RAT LIVER

by

James Woodard Kurtz

To clarify the roles of glucose and insulin in the induction of hepatic lipogenic enzymes, the contributions of these two compounds to the induction of rat hepatic glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and malic enzyme (ME) were examined.

Normal and streptozotocin diabetic male rats were starved for three days, then refed a high carbohydrate diet for four days (hereafter abbreviated S/R). In streptozotocin diabetic rats, the induction responses of G6PDH, 6PGDH, and ME proceeding from the three day starved to the four day high carbohydrate diet refed state, were 10 to 20% of those observed in similarly treated normal rats. This difference for diabetic rats was not due to inadequate glucose consumption since S/R diabetic rats consumed 59% more diet than S/R normal rats. Serum immunodetectable insulin concentrations of diabetic rats increased 2.6 fold in response to refeeding in both normal and diabetic rats. The latter insulin response did not lower serum glucose concentrations to normal levels. Supplementation of starved diabetic rats with insulin during refeeding controlled serum glucose levels

and restored the induction of G6PDH, 6PGDH, and ME to levels above normal.

Hepatocytes were isolated from 3-day starved male rats and incubated in serum-free Dulbecco's medium (DMEA). G6PDH specific activity increased 2.5-fold in 48 hour control incubations in DMEA and increased an additional 3.5-fold in the presence of 42 mU/ml insulin, 1 μM dexamethasone, and the absence of medium glucose. The effects of insulin and dexamethasone on G6PDH induction of G6PDH were dose dependent and additive. Increases in G6PDH specific activity by insulin and dexamethasone were independent of DNA synthesis. 6PGDH and ME specific activities decreased during the 48 hour control incubation in DMEA. Insulin, but not dexamethasone, prevented this decrease in activities and increased 6PGDH activity 20% above 0 hour levels. Cells incubated in DMEA with glucose in the absence of hormones showed no increase in G6PDH, 6PGDH, and ME activities. These results indicate that glucose alone is not sufficient to induce these liver enzymes and that insulin is required for the induction of G6PDH, 6PGDH and ME *in vivo* and G6PDH and 6PGDH *in vitro*.

The role of lysosomes as mediators of S/R, insulin, and glucocorticoid stimulated enzyme induction *in vivo* and in RH-35 cells was examined.

Although previously observed increases in lysosome fragility during the first hours of refeeding were confirmed, further labilization or stabilization of lysosomes did not change the induction of G6PDH and 6PGDH compared with controls. Refeeding of a high glucose diet to starved rats resulted in no significant alteration in the association of lysosomal enzyme activities with purified liver nuclei compared with activities

present in nuclei from 0 hour refed rats. Starved/refed streptozotocin diabetic rats, with or without insulin injection at doses sufficient to induce G6PDH and 6PGDH, showed no significant change in purified liver nuclear associated acid phosphatase activity throughout the experiment.

The glucocorticoid responsive system, induction of tyrosine-α-keto-glutarate transaminase in RH-35 cells in culture and in adrenalectomized rats, was used to determine if glucocorticoids elicit a nuclear translocation of lysosomes or lysosomal enzyme activity. Biochemical and electron microscopic examination revealed no change in the association of lysosomal hydrolases with nuclei of induced cells compared with those from uninduced cells. There was a 9-fold greater nuclear N-acetyl-β-D-glucosaminidase activity in RH-35 cells than in hepatocytes despite the lower total activity/DNA of RH-35 cells.

The latter results do not support the extension of C.M. Szego's hypothesis for lysosomal mediation of hormone action to include S/R, insulin, and glucocorticoid action as described.

Automated fluorometric methods developed for the analysis of DNA, protein, and selected enzyme activities: N-acetyl-β-D-glucosaminidase, G6PDH, and 6PGDH are described.

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ABBREVIATIONS

G6PDH = glucose 6-phosphate dehydrogenase

6PGDH = 6-phosphogluconate dehydrogenase

ME = malic enzyme

T_3 = triiodothyronine

TAT = tyrosine aminotransferase

ACTH = adrenocorticotrophic hormone

PTH = parathyroid hormone

TSH = thyroid stimulating hormone

FSH = follicle stimulating hormone

LH = leutinizing hormone

RH-35 = Reuber hepatoma 35

MEM = minimum essential medium (Eagle's)

FBS = fetal bovine serum

4-MU = 4-methylumbelliferone

INTRODUCTION

Organization

This dissertation is divided into four chapters, each of which is in a form acceptable for publication in biochemical journals. Chapter I was authored by James W. Kurtz and William W. Wells, published in Analytical Biochemistry 94:166-175 (1979), and is reproduced here by permission from Academic Press. Part of Chapter III (Table V) was my work and is published in the Journal of Nutrition 172:206-214 (1976) by Hartmut R. Schroeder, John A. Gauger, and William W. Wells. Chapter IV is in preparation to be submitted to the Journal of Biological Chemistry. Part I of the Appendix was presented as part of a poster entitled Lysosomal Enzymes and Liver Nuclei of the Genetically Obese Mouse authored by W.W. Wells, I.T. Mak, J.W. Kurtz, N.S. Henderson, C.A. Collins, and R.E. Ray (1979) at the XIth International Congress of Biochemistry, Toronto, Canada.

LITERATURE SURVEY

Induction of lipogenic enzymes. Lipogenesis is the successive linkage and reduction of the carbon units of acetate to form lipids. Several enzymes which are important in channeling carbons of various sources and reducing equivalents into fatty acids include: acetyl CoA carboxylase, ATP-citrate lyase, fatty acid synthetase, malic enzyme, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. The rate of lipogenesis is controlled largely through regulation of the activities of these enzymes. Control of lipid synthesis via regulation of acetyl CoA carboxylase and fatty acid synthetase has recently been reviewed (1). This survey will examine the dietary and hormonal factors which affect lipogenic enzymes with particular attention to the regulation of glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and malic enzyme (ME).

It has long been recognized that starvation lowers the activity and refeeding increases the activity of the lipogenic enzymes to levels above those of pre-starvation (termed "overshoot") (2,3,4). These early reports have been reviewed (5,6,7). The major dietary components: carbohydrate, lipid, and protein have all been examined as affecting induction of these enzymes and will be briefly reviewed in that order.

Sassoon et al. (8) reported that in normal starved/refed rats the degree of induction of G6PDH and 6PGDH was correlated with the amount of dietary glucose consumed. They also reported that a single injection of insulin (4-6 units) resulted in a 25% increase in G6PDH activity after 24 hours but concluded that dietary carbohydrate was the more important induction signal. In similar experiments from Holten's laboratory it was

found that the rates of G6PDH (9) and 6PGDH (10) synthesis were significantly correlated with the amount of dietary carbohydrate consumed. In other experiments Rudak et al., (11) found that rats fed a 60% fructose containing diet induced G6PDH to the same extent as rats fed a 60% glucose containing diet. The authors stated that since insulin release occurred in one case (glucose fed) but not the other that dietary carbohydrate was the primary signal for induction of G6DPH (see Discussion of Chapter 4 of this dissertation for a more complete analysis of this point). Derr and Zeive (12) found that infusion of a glucose-amino acid solution into starved rats for 22 hours resulted in significant elevations in G6PDH and ME activity.

Dietary lipid content has long been recognized to affect the starvation/refeeding stimulated increase in lipogenic enzymes (5). Induction of G6PDH was inversely related to the content of the fat and it was observed that unsaturated fatty acids were more effective in suppressing the induction of G6PDH than saturated fatty acids. (13). Free fatty acids (14) and their CoA esters (15) inhibit the activities of most of the lipogenic enzymes in vitro but it was suggested that, at physiological levels, this could not account for the lack of induction of dehydrogenase (14). Wolfe and Holten (16) found that fat fed rats fail to induce G6PDH because of an effect on the rate of G6PDH synthesis although effects on degradation rates were not entirely eliminated. Fatty acids may therefore regulate lipogenic enzyme activities by both short term effects on activity as well as long term effects on enzyme synthesis (17).

Dietary protein, while required for the induction of lipogenic enzymes (6,18,19), in large amounts inhibited enzyme overshoot (6). This dietary protein requirement, although not ruled out as the result of

a direct effect of specific amino acids on the control of enzyme induction, probably plays a supportive role to protein synthesis in general.

Dietary effects cannot be isolated from subsequent hormonal actions when discussing the regulation of enzyme levels *in vivo*. Several hormones including insulin, glucocorticoids and thyroid hormones have effects on the induction of the lipogenic enzymes.

Since the studies of Glock and McLean (2) it has been recognized that insulin has an effect on the liver activities of G6PDH and 6PGDH. These workers found that the liver levels of G6PDH and 6PGDH dropped during alloxan diabetes. Others have reported that insulin injection into alloxan diabetic (20,21) or normal (22) rats increased the activities of these liver enzymes. Weber and Convery (23) have reported that starved/refed alloxan diabetic rats fail to induce liver G6PDH and 6PGDH unless supplemented with insulin (4U/day). Holten *et al*. (9,10,11) have critized the finding that insulin increases G6PDH and 6PGDH activity by stating that this is a secondary effect on diet consumption. Others have stated that insulin serves to induce these enzymes by enhancing glucose transport into liver cells and glucose is then the primary signal for the induction of G6PDH and 6PGDH (24). Very recently preliminary reports have appeared using the the isolated hepatocyte system to investigate the hormonal control of G6PDH and 6PGDH (25); however no full report has yet appeared.

Glucocorticoids have been recognized as playing an important role in the starvation/refeeding stimulated induction of G6PDH and ME. Bedanier *et al*. (26-29) found, in 60% of the experiments reported, that adrenalectomized rats were unable to show a full overshoot induction of

G6PDH and ME unless supplemented with cortisol. The other 40% of the experiments showed some degree of overshoot induction in starved/refed adrenalectomized rats. Wilmer and Foster (30) found adrenalectomized rats deficient but not incapable of inducing G6PDH and 6PGDH during starvation/refeeding. When several different glucocorticoids were administered during starvation/refeeding (hydrocortisone was best), full induction was achieved.

Other laboratories studying hormonal control of induction of glucokinase in primary cultures of rat hepatocytes have reported that glucocorticoids acted synergistically with insulin to induce this enzyme (31,32). The reports that fasting (33) and glucocorticoids (34,35) decrease insulin receptor affinity for insulin would appear to be in conflict with a synergistic action of the insulin and glucocorticoids.

Thyroid hormones especially triiodothyronine, are known to regulate the activity of ME in rat liver (36,37) and in chick cells in culture (38). Very recently Towle et al. (39) demonstrated that starvation/refeeding and thyroid hormone stimulated increases in the levels of rat liver malic enzyme activity and the rate of synthesis of ME were proportional to increases in levels of mRNA for ME. Likewise, 6PGDH activity increases during starvation/refeeding were found to be proportional to changes in the levels of 6PGDH mRNA (40). In contrast, G6PDH activity increases during starvation/refeeding, while proportional to increases in the rate of G6PDH synthesis and the level of enzyme protein, could not be attributed soley to increased mRNA (41). The recent findings that T_3 is required for the insulin mediated induction of ATP-citrate lyase (42) and glucokinase (43) in primary cultures of isolated rat hepatocytes would suggest that T_3 may be of importance to the induction of G6PDH and 6PGDH

as well.

Induction of tyrosine aminotransferase by glucocorticoids. Since the initial observations by Lin and Knox (44) that glucocorticoids increase the activity of tyrosine aminotransferase (TAT) in rat liver, much has been done to elucidate the mechanism. A recent comprehensive review of glucocorticoid hormone action is available (45).

Kenney has shown by protein labeling and immunoprecipitation techniques that the increased activity of liver TAT after in vivo administration of [^{14}C]-leucine and hydrocortisone to rats is due to a specific increase in TAT synthesis (46,47). This mechanism is also supported by the work of Goldstein, Stella, and Knox (48) who showed that in the isolated, perfused rat liver, addition of puromycin and hydrocortisone to the perfusate resulted in complete disappearance of glucocorticoid mediated TAT induction. Later Hager and Kenney found that similar rapid and specific induction of TAT could be obtained in the perfused liver system by addition of glucagon or insulin to the perfusate (49).

As with the estrogen responsive systems, it was found that specific cytoplasmic (50) and nuclear (51) receptor protein fractions for glucocorticoids existed in cultured liver cells. Using intact animals, Sekeris' group showed that upon entry of [^{3}H]-cortisol into the liver, approximately 50% is associated, structurally unaltered, with cytosol, 12% is associated with both crude mitochondria and crude microsomes and 0.3% is associated with purified nuclei (52). The cytoplasmic cytosol receptor was found to have glycoprotein properties (neuraminidase and protease sensitive) with a sedimentation constant of 4S (52). In later reports, binding of [^{3}H]-cortisol to the cytoplasmic receptor glycoprotein(s) was shown to facilitate the amount of label associated with added purified

nuclei when compared with nuclei incubated with free [^{3}H]-cortisol or free [^{3}H]-cortisol plus bovine serum albumin (53). In these experiments [^{3}H]-cortisol-receptor complex formation not only enhanced nuclear binding but also increased RNA polymerase activity of nuclei. Thus the cytoplasmic receptor-steroid complex has been implicated as a functional intermediate in the induction process. Tomkins' group has shown that binding of the cytoplasmic receptor-dexamethasone complex with nuclear chromatin depends on activation. Activation can occur by incubation of the cytosol at 20°C, high (0.3 M NaCl) ionic strength, or low protein concentration (54,55) thus indicating, as in the estradiol system, that some type of structural modification must occur before binding and subsequent hormone expression. Once in the nucleus the steroid-receptor complex binds to DNA (DNase sensitive binding) receptor sites (54). Binding is then followed by increased RNA synthesis (53). The mechanism by which transcription is regulated by the receptor-steroid complex has been studied primarily by Sekeris' group (56).

To my knowledge no research has been done to elucidate the mechanism by which the glucocorticoid-receptor complex makes its way to the nucleus. Szego has proposed a mechanism for the transfer of the estrogen-receptor complex to the nucleus of extrogen sensitive target cells based on microscopic and biochemical experiments. A brief review of this evidence follows.

The lysosome as a mediator of hormone action. Comprehensive reviews of the evidence for the lysosome as a mediator of hormone action are available (57,58). Within 1-2 minutes after adminstration *in vivo* of physiological amounts of estradiol-17β, the acridine orange stained lysosomes of preputial gland and uterine cells were observed by fluorescence

microscopy to localize in and about purified nuclei (59,60). In control experiments, injections of estradiol-17α or saline did not result in accumulation of lysosomes about the nuclei. Non-estradiol target organ lysosomes were not responsive to estradiol treatment (59). These experiments demonstrate the specificity of hormone-lysosome interaction.

In different studies (61) it was observed that the *in vivo* interaction of physiological amounts of estradiol-17β with the preputial gland or uterine lysosome resulted in significant labilization of the lysosomal membrane compared with control (saline or estradiol-17α) injections. Membrane labilization was determined by a significant increase in the release of lysosomal enzymes from purified lysosomes to an isotonic or Triton X-100 incubation medium. Szego suggests (57) that the lysosomal enzymes released may in some way affect the observed structural changes of the cytoplasmic 8S estradiol-receptor complex to the nuclear 5S form (62). Pharmacologic doses of cortisol-21-acetate and propanolol just prior to estradiol-17β injections were able to reverse the previously observed: (a) metachromatic fluorescence pattern of crude and purified nuclear preparations, (b) lysosomal membrane labilization, and (c) intranuclear location of lysosomal hydrolases of preputial glands (63).

Using adrenal demedullated rats, Szego *et al.* (64) have shown that within 5 minutes after injection of low doses of the polypeptide hormone ACTH, the adrenal cortical lysosomal membranes were labilized, i.e., more susceptible to autolysis. This response to ACTH did not occur in lysosomes of non-target thyroid cells of hypophysectomized rats. Again this indicates specificity of lysosome hormone interaction. As with the observations with estrogen, ACTH treatment also resulted in the subsequent intranuclear location of lysosomal hydrolases (64). Other hormones

including: PTH, TSH, FSH, LH, epinephrine, glucagon, and cyclic AMP are proposed to act by lysosomal mediation (57).

Another report from Szego's laboratory (65) indicates the presence of an estradiol-17β receptor protein in the lysosol fraction of preputial glands (target organ for estrogens) of female rats. The lysosol fraction is the supernatant of a 105,000 x g - 1 hr centrifugation of a purified lysosome preparation which was previously incubated, with stirring, in a hypotonic buffer in 1 hour at 0-4°C. This lysosol protein (protease sensitive) posesses all of the criteria necessary for classification as a steroid receptor, i.e.: (a) target selectivity (nine-fold more receptors in preputial than liver cells), (b) stereospecificity (non-radioactive 17α-congener is unable to compete ^{3}H-estradiol-17β from the protein except at very high concentrations), (c) protein nature (protease sensitive binding), and (d) high affinity ($K_a = 10^{10}$ M^{-1}) with low capacity ($n = 10^{-13}$ moles/mg total lysosol protein) (65). In addition, this lysosol receptor protein, like the cytosol estrogen and glucocorticoid receptors, binds with greater affinity at 32°C than at 0°C. Szego speculates that a precursor-product relationship may exist.

From this accumulation of morphological and biochemical evidence, Szego has hypothesized that "...the target-specific lysosome, on activation by trophic hormone, serves as a mobile link for information transfer between the cell surface and the nucleoplasm" (57,58).

RATIONALE

The experiments performed here were designed to determine if Szego's hypothesis for lysosomal mediation of hormone action would apply to: (a) glucocorticoid hormone stimulated TAT induction in Reuber H-35 cells (Chapter II) and (b) starvation/refeeding stimulated induction of the lipogenic enzymes G6PDH, 6PGDH, and ME (Chapter III). The two major characteristics of lysosomal mediation of estradiol action reported by Szego i.e., lysosomal membrane labilization and relocalization of lysosomal enzyme activity to purified nuclei, were examined to determine if they were an integral part of glucocorticoid and starvation/refeeding stimulated enzyme induction in rat liver.

The experimental approaches taken were similar to those of Szego and were an extension of work begun in Dr. Wells' laboratory (66-68). Prior to the examination of RH-35 cells, automated fluorometric techniques for analysis of DNA, protein, and enzyme activities in samples from cell culture were developed. The presence of lysosomal hydrolases in nuclei of glucocorticoid treated RH-35 cells and adrenalectomized rats were examined biochemically and histologically. Likewise rat liver nuclei were examined for the presence of lysosomal enzyme activity during starvation/refeeding and after insulin supplementation to streptozotocin diabetic rats. Lysosome fragility was also examined as a requirement for starvation/refeeding stimulated induction of the lipogenesis enzymes G6PDH and 6PGDH.

In a final chapter, experiments using streptozotocin diabetic rats and isolated rat hepatocytes in primary culture were performed to determine the relative contributions of glucose and insulin to the induction of the lipogenic enzymes G6PDH, 6PGDH, and ME. Hepatocyte isolation and

culture techniques were developed for this purpose. The role of glucocorticoids and triiodothyronine were also examined in the isolated hepatocyte system.

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Automated Fluorometric Analysis of DNA, Protein, and Enzyme Activities: Application to Methods in Cell Culture[1]

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Automated fluorometric methods for the analysis of DNA, protein, and selected enzyme activities for *N*-acetyl-β-D-glucosaminidase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase are described. Instrumentation for these assays includes a Gilford 3500 computer-directed analyzer in conjunction with a Farrand Ratio Fluorometer-2 modified for flowthrough sampling. Comparisons were made between the automated fluorometric methods described and manual spectrophotometric or fluorometric methods for reproducibility, speed of analysis, and quantitative correlation. Typical values of *N*-acetyl-β-D-glucosaminidase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase activities obtained by these methods in isolated rat hepatocytes and Reuber H-35 hepatoma cells are reported.

Studies using cell culture methodology often require the use of microassays. While manual fluorometric methods can provide the sensitivity needed, they are often accompanied by reading fluctuation or background noise which is a combination of a number of factors, including type and handling of sample, temperature, and fluorometer characteristics (1,2). This paper describes the development of assay methods for representative cellular components using an automated fluorometer which eliminates much of the experimental error previously encountered.

MATERIALS AND METHODS

Sample preparation. Reuber H-35 hepatoma cells (RH-35 cells) were obtained from Dr. Richard Hanson, Temple University, Philadelphia, Pennsylvania. Monolayer cultures were grown in 75-cm² plastic tissue culture flasks (Bioquest, Oxnard, Calif.) in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells were grown in Eagle's minimum essential medium (MEM)[3] with Earle's salts prepared from powder (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10^5 U/liter potassium penicillin G, 0.1 g/liter streptomycin (Chas. Pfizer and Co., Inc., New York, N. Y.), and 10 g/liter sodium bicarbonate and sterilized by filtration through a 0.45-μm filter (Millipore Corp., Bedford, Mass.). After filtration, the medium was tested for sterility for 1 week by innoculation into sterile aqueous solutions of 2.4% thioglycolate and 3.7% brain-heart infusion (Difco Laboratories, Detroit, Mich.). Before use, Eagle's MEM was further supplemented with nonessential amino acids and 10% fetal calf serum (Grand Island Biological Co.). Each T-flask yielded approximately 4×10^7 cells at near confluency.

[1] This work was supported by U. S. Public Health Service Grant AM 10209.

[2] To whom inquiries should be addressed.

[3] Abbreviations used: MEM, minimum essential medium (Eagle's); PBS, phosphate-buffered saline; EB, ethidium bromide; NAGase, *N*-acetyl-β-D-glucosaminidase; G6PDH, glucose 6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; 4-MU, 4-methylumbelliferone.

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Rat liver hepatocytes were obtained from 200-g male Holtzman rats by the two-step method of Seglen (3). Magnesium-free Krebs–Henseleit bicarbonate buffer (4) gassed with hydrated 95% O_2/5% CO_2 was used. Calcium was absent during preperfusion and present during collagenase perfusion.

Normal hepatocytes or RH-35 cells were suspended in 4 ml of ice-cold 0.32 M sucrose and 3 mM $MgCl_2$ to a density of approximately 10^7 cells/ml. They were homogenized for 60 s in 18 × 104-mm glass test tubes using a Tekmar homogenizer (Tekmar Co., Cincinnati, Ohio) equipped with a small probe (10 EN) and operated at 87 V. Three milliliters of this homogenate were centrifuged at 800*g* for 10 min. To the 800*g* pellet was added 1.1 ml of 0.32 M sucrose and 3 mM $MgCl_2$, and 1.1 ml of resuspended pellet was used to obtain purified nuclei as described by Szego and Seeler (5). The 800*g* supernatant (2 ml) was again centrifuged at 22,000*g* for 15 min and this supernatant fraction was designated F.

Instrumentation. Assays were performed using the Gilford System 3500 computer-directed analyzer (Gilford Instrument Co., Inc., Oberlin, Ohio) modified so that the reading signal originated from a Farrand Ratio Fluorometer-2 (Farrand Optical Co., Inc., Valhalla, N. Y.) instead of the standard Gilford 300-N spectrophotometer (Figs. 1 and 2). A thermostated, vacuum-operated, flow-through cuvette (70 μl), specially designed and available through the Gilford Instrument Company, was inserted in place of the standard Farrand cuvette holder

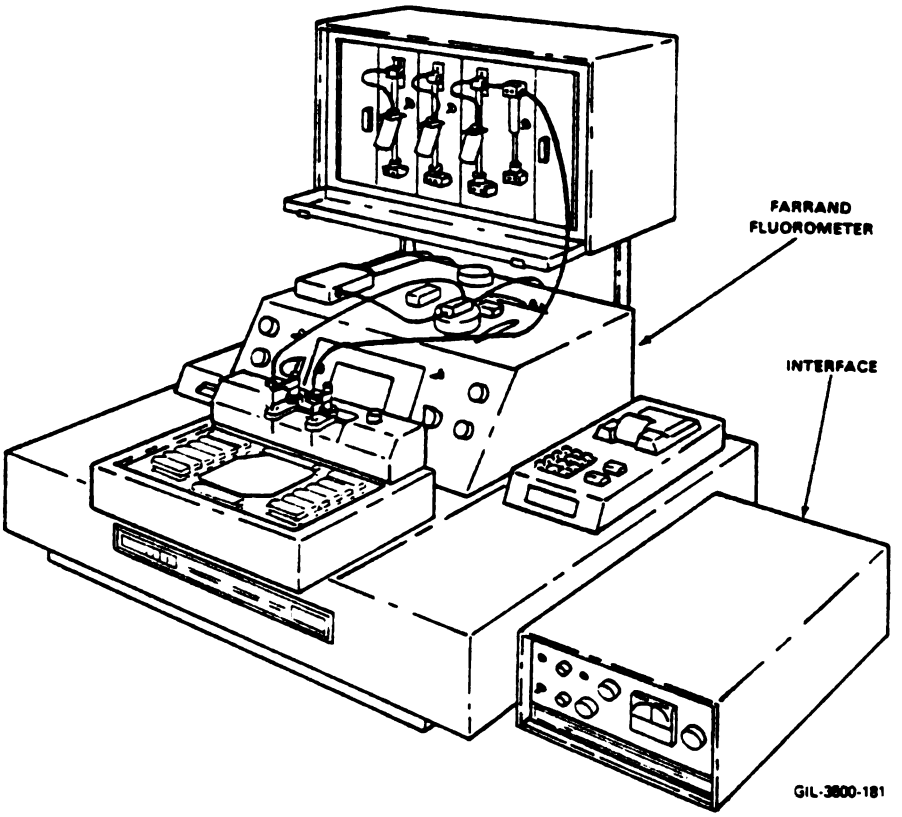

FIG. 1. Gilford Model 3500 computer-directed analyzer interfaced with the Farrand Ratio Flurometer-2.

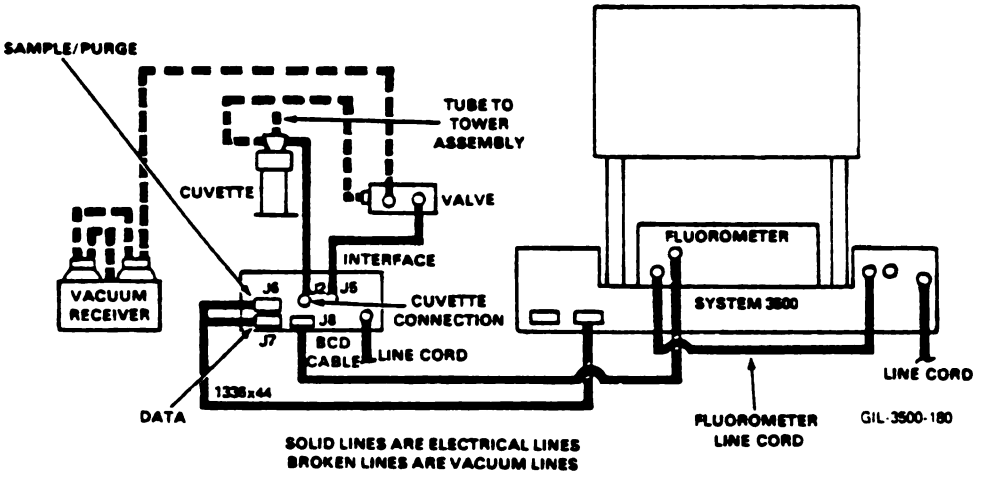

FIG. 2. Electrical and vacuum interconnections of the 3500 fluorescence system.

(Fig. 3). The Gilford Model 3500 computer-directed analyzer carries out programs directed by magnetic cards specified for standard methods in clinical chemistry. The most versatile cards for research are designated as Kinetics 1 and 2 and Endpoints 1 and 2. For the general kinetics programs, a keyboard permits the operator to select any desired time interval. The utility of the system for the research laboratory can be extended by the availability of special programs obtained from the Gilford Instrument Laboratories, Inc. A series of integrated functions automatically conduct the measurements, store the data, and print out the results in appropriate units. For a fuller description of Model 3500 hardware, e.g., transporter, pipetter/diluter, dispensers, and Farrand Ratio Fluorometer-2 to Gilford 3500 interface system, contact the Gilford Instrument Laboratories, Inc. These accessories to the standard Gilford 3500 and the Farrand Ratio Fluorometer-2 are available through the Gilford Instrument Laboratories, Inc. All assays were carried out in plastic strips (Gilford Instrument Co., Inc.) containing four reaction cups per strip. In most cases standards and samples were pipetted manually into the reaction cups, and the strips were kept on ice until initiation of the reaction. For experimental programs, we have found that adequate reagent mixing is obtained when the volume of added reagent is equal to or greater than the volume of sample to which it is added. The calibration sequence of standards (see Table 1) used in all analyses allows for the adjustment of the fluorometer such that all readings are on scale at one range setting. Filters and fluorometer settings used for all analyses are listed in Table 2.

DNA analysis. DNA analysis was performed following the procedure of Karsten and Wollenberger (6,7). Reagents I and II were freshly made by mixing component stock solutions. Reagent I is 12.5 μg/ml RNase (Sigma Chemical Co., St. Louis, Mo.: type 1-A) and 4.13 IU/ml heparin (Sigma, type 1) dissolved in phosphate-buffered saline (PBS). PBS was made according to the method of Karsten and Wollenberger (6). Reagent II is 16.7 μg/ml ethidium bromide (Sigma) in PBS. Calf thymus DNA (Sigma, type I), 0.1 mg/ml in PBS, was used as a standard. All stock solutions were stored at 4°C and were stable for at least 1 month.

Purified nuclei are assayed for DNA by the Endpoint 1 and Bilirubin-direct programs using the sequence of runs 1 and 2a (Table 1). Run 1 achieves the mixing of up to

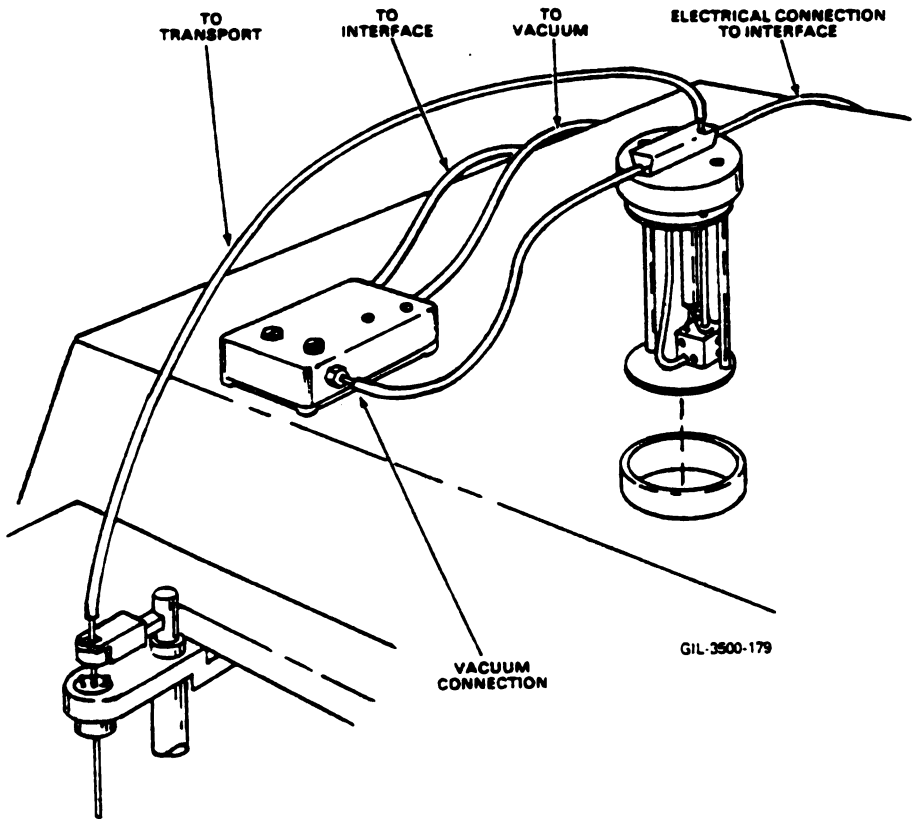

FIG. 3. Thermostated cuvette and valve connections of the Farrand Ratio Fluorometer-2/Gilford 3500 fluorescence system.

0.2 ml of standard or sample with 1 ml of a RNase/heparin mixture (reagent I) in reaction cups. After run 1 the reaction strips are removed from the Gilford 3500 rack transporter and incubated 20 min at 37°C in a water bath and 15 min at room temperature. Following incubations, run 2a accomplishes the mixing of 1.5 ml of the ethidium bromide solution (reagent II) with the samples and the reading of fluorescence. The final concentration of ethidium bromide is 10 μg/ml.

The assay of whole homogenate fractions for DNA is similar to that of purified nuclei except that the high fluorescence due to whole homogenate alone necessitates the inclusion of sample blanks (runs 1 and 2b). Run 1 is the same as in the assay of purified nuclei except that samples and standards are pipetted in two sets. The first set of samples serve as blanks and the second set of samples as complete reactions. After a 30-min incubation at 37°C and a 15-min incubation at room temperature, run 2b is started. In this run, 1.5 ml of PBS is mixed with the first set and reagent II is mixed with the second set of samples and standards. Fluorescence readings are automatically made in this run exactly 2.5 min after each mixing.

Slopes, intercepts, and correlation coefficients of standard curves are obtained by least-squares analysis. Calculation of the concentration of DNA in mg DNA/ml sample is as follows.

Purified nuclei: $(A - C)/(E)F$.

Whole homogenate: $[(A - B) - (C - D)]/(E)F$.

where

A = fluorescence of sample + RNase + heparin + PBS + ethidium bromide (EB) + EB–DNA complex,
B = fluorescence of sample + RNase + heparin + PBS,
C = fluorescence of RNase + heparin + PBS + EB,
D = fluorescence of RNase + heparin + PBS
E = slope of standard curve ($\Delta F/\Delta$mg DNA),
F = undiluted sample volume (ml).

For comparison, DNA was assayed manually by the diphenylamine method of Giles and Myers (8). Diphenylamine (Mallinckrodt, St. Louis, Mo.) was twice recrystallized from 100% ethanol, mp 52.8–54°C. Calf thymus DNA, 0.1 mg/ml in PBS, was used as a standard. After the 18-h incubation, samples were centrifuged 15 min at 1146g at room temperature, and the absorbance of the supernatant solutions was measured at 595 and 700 nm.

Protein analysis. Protein analysis was performed by a modification of the method of Böhlen *et al.* (9). Reagent III is 0.2 M borate buffer, pH 9.25 (boric acid solution titrated with NaOH), and reagent IV is fluorescamine (Fluram Roche Diagnostics, Nutley, N. J.) at a concentration of 30 mg/100 ml in $CaSO_4$-dried acetonitrile. Bovine serum albumin (Sigma) was used as a standard. Reagents III and IV can be stored indefinitely in the dark at room temperature.

TABLE 1

ASSAY PROTOCOLS[a]

Assay	Program	Run[b]	Reagent addition: Tower	Reagent addition: Reagent	Reagent addition: Volume (ml)	Standards and samples: Reaction cup No.	Standards and samples: Contents[c]	Standards and samples: Final sample volume (ml)	Standards and samples: Total assay volume (ml)
DNA	Endpoint 1 (mode 1)	1	A	I	1	1–4	Calibration sequence	0.2	2.7
						5–12	Standard curve (0–15 μg DNA)		
						13–end	Sample		
	Bilirubin-Direct	2a	A	II	1.5				
	Bilirubin-Direct	2b	A	PBS 1st set	1.5				
				II 2nd set	1.5				
Protein	Bilirubin-Direct	1	A	III	1.5	1–4	Calibration sequence	0.1	2.1
			B	IV	0.5	5–12	Standard curve (0–12 μg protein)		
						13–end	Samples		
NAGase	Endpoint 1c (mode 1)	1	A	V	0.25	1–4	Calibration sequence	0.02	2.27
						5–12	Standard curve (0–1.5 nmol 4-MU)		
						13–end	Samples		
	Endpoint 1c (mode 3)	2	A	VI	2.0				
G6PDH and 6PGDH[d]	Kinetics 1	1	A	VII	2.0	1–4	Calibration sequence	0.1	2.1
						5–12	Standard curve (0–20 nmol NADPH)		
						13–end	Samples		
	Kinetics 1	2	A	VIII	2.0				

[a] See Materials and Methods for a description.
[b] A run is defined as the handling of samples and reagents in a programmed sequence.
[c] The calibration sequence is comprised of reaction cups 1–4 containing the lowest standard in cups 1, 2, and 4, and the highest standard in cup 3.
[d] Preincubation time = 1 s, incubation time = 20 s, and integration time = 10 s.

TABLE 2

FLUOROMETER FILTERS AND SETTINGS[a]

	DNA	Protein	NAGase	G6PDH, 6PGDH
Excitation wavelength (nm)	360	390	366	340
Filter number[b]	(7-60)	(7-54)	(7-60)	(7-60)
Emission wavelength (nm)	590	475	450	460
Filter number	(3-69)	(3-72)	(3-73)	(3-72)
Excitation slit setting	4	1	1	3

[a] In all assays, the following instrument settings were used: damping = lo, mode = sample, range = 1, temperature = 25°C, sample time = 5, vacuum = 10, and multiplication factor = 100.
[b] Farrand Optical Co., Inc., Valhalla, N. Y.
[c] Farrand Ratio Fluorometer-2.

Whole homogenate, purified nuclei, and F fractions were assayed as indicated in Table 1 using the Gilford 3500 program for direct bilirubin analysis. The instrument pipetting system forcefully mixes standard or sample with 0.5 ml fluorescamine solution and 1.5 ml borate buffer, pH 9.25, and the fluorescence is measured exactly 2.5 min after each mixing. The ordinate-intercept of the standard curve served as the blank since these samples do not contribute significantly to the fluorescence of the product.

For comparison, protein was also determined manually by the procedure of Lowry *et al.* (10) using bovine serum albumin as standard.

N-acetyl-β-D-glucosaminidase analysis. *N*-Acetyl-β-D-glucosamindase (NAGase) activity was determined by a method modified from that of Robins *et al.* (11) using the Endpoint 1c program as summarized in Table 1. The aqueous 75 μM 4-methylumbelliferone (Sigma) standard used for preparation of standards listed in Table 1 was stable at −80°C for at least 6 months. Assays of whole homogenate and F fractions were initiated (run 1) by the mixing of up to 0.05 ml sample or standard with 0.25 ml of the assay mix, reagent V. Reagent V, made on the day of assay from component stock solutions, was: 50 mM sodium citrate, pH 4.3, 0.2% (w/v) Triton X-100 (Research Products International Corp., Elk Grove Village, Ill.), and 1.03 mM 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Sigma). After a timed, room temperature incubation period (usually 15 to 20 min), reactions were stopped (run 2) by the addition of 2.0 ml of 20 mM 2-amino-2-methyl 1-propanol (Sigma), pH 10.35, reagent VI. The fluorescence of each sample was measured in run 2, 9 s after addition of reagent VI. In the calculation of *N*-acetyl-β-D-glucosaminidase activity, the ordinate-intercept of the standard curve served as the blank since sample alone does not contribute significantly to the overall fluorescence. In addition, manual assays were performed by the same procedure as described for the automated method except that reactions and fluorescence readings were performed in 10 × 75-mm test tubes.

Glucose 6-phosphate and 6-phosphogluconate dehydrogenase analysis. The procedure used for the determination of glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) is a modification of that used by Rudack *et al.* (12) and is summarized in Table 1. An appropriate volume of reagent VII is freshly prepared by mixing stock solutions to give: 115 mM Tris, pH 8.0, 10 mM $MgCl_2$, 50 μM $NADP^+$, 0.6 mM 6-phosphogluconate, and 2 mM glucose 6-phosphate (all organic chemicals from Sigma). Reagent VIII is identical to reagent VII except that glucose 6-phosphate is omitted. A 1 mM NADPH (Sigma) solution, pH 10, is pipetted manually into the reaction cups to give the various amounts of standard indicated in Table 1. For comparison, dehydrogenase assays were also performed with the same assay mixture using a Gilford 2400-S recording spectrophotometer (Gilford Instrument Co., Inc.). $NADP^+$ and NADPH solutions were stored at −80°C; all other stock solutions were stored at 4°C.

Using the Kinetics 1 program, the combined glucose 6-phosphate and 6-phosphogluconate dehydrogenase activity are measured during run 1, and 6-phosphogluconate dehydrogenase activity alone is

measured during run 2. Glucose 6-phosphate dehydrogenase activity is calculated as the difference between the rates obtained in runs 1 and 2. Assays on F fractions are performed at 30°C.

Statistical methods were employed according to Steel and Torrie (13).

RESULTS

DNA Analysis

Figure 4 presents DNA standard curves for the ethidium bromide procedure performed either manually or by the automated method. To evaluate the reproducibility of the two procedures for analysis of DNA, the coefficients of variability (*CV*) of the regression coefficients (slopes) of either fluorescence or $A_{590}-A_{700}$ vs the concentration of DNA were determined for a range of 1 to 15 μg of DNA. These results, presented in Table 3, indicate that the automated method is more reproducible than the manual method. By increasing the fluorometer sensitivity 10-fold, as little as 0.1 μg of DNA could be detected; however, reading variability was ±14%. Recovery of standard amounts of DNA added to samples of whole homogenate was 98%. A comparison of the diphenylamine and ethidium bromide methods for the analysis of DNA in whole homogenate and purified nuclei fractions revealed no difference (analysis of variance) in quantities of DNA determined when fresh or once-frozen samples were analyzed (data not shown). A comparison of the time efficiency of performing the automated and manual methods, Table 4, shows the automated method to be roughly 1.7 times more rapid. This comparison does not include the 18-h room temperature incubation necessary in the manual method.

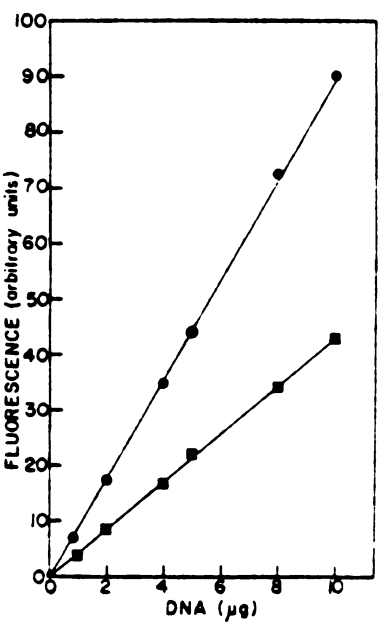

FIG. 4. Comparison of fluorescence obtained from the assay of standard amounts of calf thymus DNA by the ethidium bromide method described when performed manually or automatically. Manual assays (●) were performed by the same procedure as described for the automated procedure (■) except that reactions and fluorescence readings were carried out in 10 × 75-mm test tubes.

TABLE 3

COMPARISON OF REPRODUCIBILITY OF AUTOMATED AND MANUAL METHODS USING THE COEFFICIENTS OF VARIABILITY OF REGRESSION COEFFICIENTS

	Method[a]			
	Automated		Manual	
Analysis	*CV* (%)	*n*	*CV* (%)	*n*
DNA[b]	1.02	44	1.47	48
Protein[b]	0.92	47	1.14	33
NAGase[c]	0.71	12	1.55	12
6PGDH[c]	2.03	10	5.30	8
G6PDH[c]	13.4	8	13.6	6

[a] *CV*, coefficient of variability; *n*, number of analyses.

[b] Regression analysis of standard curves.

[c] Regression analysis of enzyme velocity vs sample volume.

Protein Analysis

Determination of protein concentrations in four different preparations of rat hepatocyte fraction F (1–4 mg protein/ml) by both

TABLE 4

PRODUCTIVITY COMPARISON OF THE MANUAL AND AUTOMATED METHODS DESCRIBED

	Method[a]	
	Automated	Manual
DNA	44	26
Protein	56	30
NAGase	75	50
G6PDH and 6PGDH	67	48

[a] Rates (analyses/h) include sample pipetting time.

the manual Lowry and the automated fluorescamine methods gave similar results (no difference by analysis of variance).

A comparison of the coefficients of variability of regression coefficients for standard curves of the manual Lowry protein assay with the automated fluorescamine assay (Table 3) shows that the latter method was slightly more reproducible than the manual Lowry method. A comparison of the sensitivity of these methods in analyzing the same amount of protein as determined by the ratio of slopes of standard curves for each method, indicated that the fluorescamine method was 4.3 times more sensitive than the Lowry method. Table 4 shows that the speed of performing analyses by the automated method is nearly double that of the manual method.

N-Acetyl-β-D-Glucosaminidase Analysis

Quantitative agreement between the manual and automated methods for this assay was obtained (analysis of variance). Using the automated method described, the linearity of *N*-acetyl-β-D-glucosaminidase activity in isolated rat hepatocyte fraction WH with time and sample volume was verified with samples having activities up to 1 nmol 4-methylumbelliferone per minute. A comparison of the coefficients of variability of the regression coefficient for varying amounts of sample vs *N*-acetyl-β-D-glucosaminidase activity (Table 3), determined either automatically or manually, revealed the former method to be more than twice as reproducible as the latter. A comparison of time efficiency for the automated and manual methods (Table 4) indicated that the automated method was 1.5 times more efficient than the manual method. Typical values of *N*-acetyl-β-D-glucosaminidase activity in WH fractions of rat hepatocytes and RH-35 cells are shown in Table 5.

Glucose 6-Phosphate and 6-Phosphogluconate Dehydrogenase Analysis

Quantitative agreement between the manual and automated methods for these assays were obtained (analysis of variance). Using the automated method, dehydrogenase activity increased linearly with the volume of fraction F assayed up to an activity of 15 nmol NADPH per minute. In this continuous assay, linearity of activity with time was determined for every sample using the Kinetics 1 program. This program dictates the storage of fluorescence readings

TABLE 5

ENZYME ACTIVITIES OF RAT LIVER CELLS[a]

Sample	NAGase[b]	G6PDH[c]	6PGDH[c]
Rat hepatocytes	408.6 ± 55.8 (4)	36.7 ± 28.3 (4)	94.8 ± 20.9 (4)
RH-35 cells	139.0 ± 59.0 (5)	48.0 ± 15.0 (8)	28.1 ± 5.2 (8)

[a] *N*-Acetyl-β-D-glucosaminidase activities are from whole homogenate fractions and dehydrogenase activities are from 22,000*g* supernatant fractions (F).

[b] nmoles 4-MU min^{-1} mg DNA^{-1}.

[c] nmoles NADPH min^{-1} mg $protein^{-1}$.

(7 per second) for three operator-specified intervals of time and then calculates ΔF per minute and percent deviation from linearity for each analysis. The described assays seldom accelerated or decelerated more than 1% from a linear rate. Comparison of the coefficients of variability of the regression coefficients of varying amounts of sample vs enzyme activity, determined by the automated and manual methods described (Table 3), indicate that the automated methods are equal to or more reproducible than the manual methods for glucose 6-phosphate and 6-phosphogluconate dehydrogenase activity determinations respectively. As shown in Table 4, use of the automated method resulted in the processing of samples 1.4 times more rapidly than spectrophotometric analysis using a Gilford 2400-S. Typical values of the dehydrogenase activities of fraction F from isolated rat hepatocytes and RH-35 cells are shown in Table 5.

DISCUSSION

While we have found the diphenylamine method of Giles and Myers (8) for analysis of DNA entirely adequate for rat liver, isolated rat hepatocytes, and RH-35 cells, the automated ethidium bromide method described gives comparable results and is more rapid and slightly more reproducible. We therefore agree with Karsten and Wollenberger (7) that the ethidium bromide assay is the method of choice for tissue culture work. In the assay of whole homogenate, samples were somewhat turbid; however, adequate correction for this could be made by the inclusion of appropriate blanks. Therefore, fluorometry by surface illumination is not necessary and right-angle illumination can be used. This is in agreement with the findings of others (7,14,15). It was also found that the extensive heating (50°C, 1 h) recommended by van Dyke and Szustkiewicz (16) was not needed and that incubation at 37°C for 20 min as indicated by Karsten and Wollenberger (6,7) was sufficient. The lower fluorescence per microgram DNA in the automated method compared with readings obtained with the manual method (Fig. 4) is due to the smaller size of the flow-through cuvette. This does not present a problem in routine assay since gain adjustments are made to give a standard to blank ratio of 30 for 10 μg DNA. While quantities less than 1 μg of DNA can be measured, they are accompanied by a corresponding increase in reading variability. Although unnecessary for these analyses, greater sensitivity could be achieved with this system by use of a time-averaging program.

Analysis of isolated rat hepatocyte fraction F for protein by the automated fluorescamine method described gave results which were similar to those obtained by the Lowry method. In addition, the method described is much simpler, faster, and as reproducible as the Lowry method over a range of 0.5 to 50 μg of protein. The limit of protein detected by the automated fluorescamine method described is 0.5 μg. This sensitivity has proved adequate for analyses of isolated rat hepatocyte and RH-35 cell fractions.

The ability to precisely control the timing of multiple reagent additions to large numbers of samples made the described system particularly useful for endpoint assays of enzyme activities. This was illustrated by the highly reproducible measurement of *N*-acetyl-β-D-glucosaminidase activity (Table 3). The values of *N*-acetyl-β-D-glucosaminidase in isolated rat hepatocytes reported in Table 5 (408.6 ± 55.8 nmol 4-MU min^{-1} mg DNA^{-1}) are slightly lower than those previously reported for whole liver [500–3,000 nmol mm^{-1} mg DNA^{-1} (17,18), assuming 2.4 mg DNA/g fresh tissue (19)] owing to differences in substrates and cell types. Automated methods for the measurement of several other lysosomal enzymes are available (20).

The values obtained for isolated rat hepatocyte G6PDH and 6PGDH (Table 5) using

the fluorometric method compare well with results obtained in rat liver by this and other laboratories (12,21,22). The elevation of G6PDH activity in the RH-35 tumor cell line compared with levels in isolated rat hepatocyte (Table 4) is consistent with the findings of Selmeci and Weber (23).

For the analysis of DNA, protein, and the activities of the representative enzymes, *N*-acetyl-β-D-glucosaminidase, glucose 6-phosphate, and 6-phosphogluconate dehydrogenase in isolated rat hepatocytes or RH-35 cells, the methods described are as simple and reliable as the more conventional assay methods. The combined automated and fluorometric aspects of the methods described provide for the rapid and reproducible handling of increased numbers of samples derived from relatively small amounts of tissue or cultured cells.

ACKNOWLEDGMENT

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Chapter II

Induction of Tyrosine Aminotransferase in RH-35 Cells and in Rat Liver. Investigation of the Involvement of Lysosomes.

ABSTRACT

Lysosomes have been implicated in the process of translocation of the estradiol-receptor complex to nuclei of target tissues shortly after exposure to this hormone (Szego, C.M. (1974), see reference 2). The glucocorticoid responsive system, induction of tyrosine aminotransferase (TAT) in RH-35 cells in culture and in adrenalectomized rats, was used to determine if glucocorticoids elicit a similar nuclear translocation of lysosomes or lysosomal enzyme activity.

TAT activity of confluent monolayer cultures of RH-35 cells incubated with 1 μM dexamethasone (optimal dose) increased to a maximum of 8-fold above control after 8 hours of incubation. Hydrocortisone (2 μM) produced a slightly lower (3-fold) increase in activity in a 5 hour incubation. Isolated nuclei were examined for the lysosomal hydrolase activities: acid phosphatase, N-acetyl-β-D-glucosaminidase, cathepsin D, and β-glucoronidase at various times after addition of 1 μM dexamethasone to cells. In separate studies, RH-35 cells were stained for acid phsophatase and examined by electron microscopy at various times after addition of 1 μM dexamethasone. These experiments revealed no consistent change in the association of lysosomal hydrolases with nuclei of induced cells

compared with those from control cells. An analogous *in vivo* experiment was performed by injecting an inducing dose of hydrocortisone (10 mg/100 g body weight) into adrenalectomized rats. Nuclear acid phosphatase activities were lower after adrenalectomy and were further decreased or unchanged at early times after hydrocortisone injection. Comparison of RH-35 cell and normal isolated hepatocyte nuclear N-acetyl-β-D-glucosaminidase activities revealed a 9-fold greater association of activity with RH-35 cell nuclei despite lower RH-35 cell activity per cellular DNA.

The results presented here do not support the extension of Szego's hypothesis to include glucocorticoid action. The possibility remains that such a phenomenon occurred but at a magnitude not detectable by the present methods.

INTRODUCTION

The current understanding of glucocorticoid hormone action has recently been thoroughly reviewed (1). One aspect of glucocorticoid action about which little is known is the means by which the hormone-receptor complex migrates through the cytosol to the nucleus. Szego has proposed a mechanism for the transfer of the estrogen-receptor complex to the nucleus of extrogen sensitive-target cells (2). Within 1 to 2 minutes after administration *in vivo* of physiological amounts of 17β-estradiol, the acridine orange stained lysosomes of preputial gland and uterine cells were observed, by fluorescence microscopy, to localize in and about nuclei (3, 4). Control injections of 17α-estradiol were ineffective in eliciting this response and non-target tissues were unresponsive to 17β-estradiol (3). Nuclei purified from these tissues by conventional procedures showed 15-fold increases in the lysosomal: acid phosphatase, β-glucuronidase, and acid ribonuclease II within 15 min of 17β-estradiol injection (4). Examination of lysosome enriched fractions from preputial gland cells of ovariectomized rats revealed the presence of estradiol receptor proteins (5).

The present investigation was designed to examine the possibility that lysosomes are involved in the translocation of the glucocorticoid-receptor complex to the nuclei. Induction of TAT in RH-35 cells was the hormone responsive system chosen because translocation of the hormone-receptor complex to the nucleus is well documented as an integral part of the induction of TAT (1).

MATERIALS AND METHODS

Cell culture conditions. Reuber H-35 hepatoma cells obtained from Dr. Richard W. Hanson were grown in MEM containing 10% FBS and normal amounts of non-essential amino acids as previously described (6). During culture, medium was changed every four days. Eighteen hours prior to experiments, FBS-containing medium was removed, the cells were rinsed once with FBS-deficient medium then replenished with 10 ml FBS-free medium. Experiments were, as indicated, begun either by changing medium to one containing the experimental conditions or by addition of a concentrated stock solution of the experimental compounds to incubation medium. In initial studies, cells were detached from the surface of the T-flasks by a 6 min trypsinization at 37°C in an atmosphere of 95% air/5% CO_2 using a 0.25% trypsin in a solution containing (per liter): 1 g glucose, 8 g NaCl, and 0.4 g KCl. The cell suspension was then decanted into a graduated conical centrifuge tube and centrifuged 4 min at 3/4 full speed in a table top centrifuge (IEC, Model HN), washed once with 6 ml 0.32 M sucrose containing 3 mM $MgCl_2$. The cell pellet was then brought to 3 ml with 0.32 M sucrose, 3 mM $MgCl_2$ and homogenized and centrifuged as described previously (6) to obtain 800 x g-10 min and 22,000 x g-15 min supernatants and purified nuclei. In later experiments, cells were washed twice with 0.32 M sucrose, 3 mM $MgCl_2$ while attached to the flasks and harvested in 0.32 M sucrose, 3 mM $MgCl_2$ with a rubber policeman in a final volume of 3 ml. Homogenization and fractionation were then performed as indicated above.

Analysis of enzyme activity, DNA, and protein concentration. Tyrosine aminotransferase (EC 2.6.1.5) activity was determined

according to the procedure of Granner and Tomkins (7) in the presence of 2 mM diethyl-dithiocarbamate which is used to inhibit breakdown of the endproduct, p-hydroxyphenylpyruvate, by p-hydroxyphenylpyruvate oxidase. Due to a slight drift in the absorbance of p-hydroxyphenylpyruvate (A_{331nm}) with time, readings were taken at 60 min after stopping reactions. Assays were begun by the addition of sample to complete reaction mixtures pre-incubated to 37°C, then stopped by addition of KOH as indicated (7). The pH optimum for RH-35 cell TAT was determined to be 7.6. One unit (U) of activity is 1 μmole p-hydroxyphenyl pyruvate formed per minute.

Cathepsin D (EC 3.4.23.5) was determined by the [^{3}H]-acetyl hemoglobin method of Barrett (8) with slight modification. The substrate, [^{3}H]-acetyl hemoglobin, was prepared according to the procedure of Barrett (8) and had a specific activity of 0.02 μCi/mg soluble hemoglobin. The reaction mixture contained 3.6 mg of [^{3}H]-hemoglobin, 0.24 M formate buffer, pH 3.0, 0.1% triton X-100 and sample and water to a final volume of 210 μl. Assays were started by the addition of sample (86 μl) to the assay mixture which has been pre-incubated 5 min at 45°C. Incubations were 90 min at 45°C and were stopped by the addition of 1 ml 3% (w/v) TCA at 4°C. The samples were centrifuged at 1100 x g for 15 min. Aliquots (0.5 ml) of the supernatant were counted in Bray's solution (9) in a Beckman CPM-100 liquid scintillation spectrometer. The pH optimum for this enzyme in RH-35 cells was determined to be 3.0. Activity is expressed as TCA soluble tritium cpm per mg DNA in a 90 min. incubation.

β-glucuronidase (EC 3.2.1.31) activity was determined at 25°C using the fluorometric method of Robins et al. (10). The pH optimum of this enzyme in RH-35 cells was determined to be 3.6.

N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) (hexosaminidase) activity and protein and DNA concentrations were determined fluorometrically as previously described (6). For both β-glucuronidase and hexosaminidase activities, one unit (U) is 1 μmole 4-methylumbelliferone released per minute under the assay conditions.

Electron microscopy. At various times after addition of dexamethasone to confluent T-flasks of RH-35 cells, medium was decanted and cells were fixed with 5 ml of 2% glutaraldehyde (10% stock glutaraldehyde from Electron Microscopy Sciences diluted 1/5 with 0.1 M Na cacodylate, pH 7.2, just before use). While cells were being fixed they were scraped from T-flasks into 10 x 1 cm test tubes and incubated at room temperature a total of 20 min. Cells suspensions were centrifuged as described above for cell harvesting and washed once with 0.15 M Na acetate buffer, pH 5.0. Cells were then resuspended in Gomori acid phosphatase stain (11) and incubated for 40 min at 37°C in the absence and presence of 0.01 M NaF which inhibits acid phosphatase and therefore served as a control for acid phosphatase staining. Following this incubation, cells were washed twice with 0.4 M sodium acetate buffer, pH 5.0 to remove non-specific staining. Cells were dehydrated by washing consecutively in 25, 50, 75, and 95% ethanol solutions, 10 min in each and finally for 30 min in 100% ethanol. Samples were then given to June Mack of the electron microscopy facility of the Pesticide Research Center, Michigan State University. Cells were embedded in Epon-Araldite and ultrathin sectioned with a Sorvall MT-2 ultramicrotome. Sections were stained with saturated uranyl

acetate and post-stained in a saturated lead citrate solution. Cells were then examined using a Philips 300 transmission electron microscope.

In vivo studies. Male Holtzman rats 180-200 g were adrenalectomized or sham operated according to the procedure of Zarrow et al. (12) and given lab chow and 1% NaCl as a drinking solution, ad libitum. Four days after adrenalectomy, rats were starved for 19 h then injected with hydrocortisone 21-Na succinate (10 mg/100 body weight) or saline and sacrificed by decapitation at various times after injection. Livers were rapidly removed and placed in 0.32 M sucrose 3mM $MgCl_2$ at 0-4°C. Four grams of liver were minced with scissors then combined with 23 ml 0.32 M sucrose 3 mM $MgCl_2$ and homogenized 30s at 65V with a Tekmar homogenizer. The whole homogenate was filtered through 4-ply cheese-cloth and centrifuged at 800 x g for 10 min. The 800 x g supernatant was then centrifuged for 15 min at 22,000 x g to obtain a supernatant containing free lysosomal enzyme activity. The entire 800 x g pellet was mixed with 20 ml of 2.4 M sucrose and 1 mM $MgCl_2$ and centrifuged for 45 min at 30,000 rpm in a Beckman L-2 ultracentrifuge with a 30K rotor. The purified nuclear pellet was carefully resuspended in 1.5 ml 0.32 M sucrose 3 mM $MgCl_2$. DNA analysis was performed by the method of Giles and Meyers (13) as described previously (6).

RESULTS

Development of the tyrosine aminotransferase induction system. Reuber H-35 cells grew in MEM containing 10% FBS at a logarithmic rate as shown in Figure 1. Doubling time for the cells during the logarithmic phase of growth was 24 hours. Cells were routinely innoculated at a density of 10^6 cells per flask and used in experiments 1 week later a density of 4×10^7 cells per flask. At this stage, cells were in a confluent monolayer and, as indicated in Figure 1, not rapidly replicating. The induction of TAT in RH-35 cells incubated in the presence of dexamethasone is demonstrated in Figures 2 and 3. As shown in Figure 2, the optimum dose of desamethasone for the induction of TAT was approximately 1 μM. TAT activity increased rapidly during the first five hours after addition of dexamethasone (Figure 3) then leveled by 8 hours at an 8-fold elevation above control activity and began to decline slightly after 10 hours of incubation. During the same time period, the activity of TAT remained constant in cells incubated in the absence of dexamethasone. RH-35 cells were also responsive to hydrocortisone. In experiments not shown here, TAT specific activity increased 3-fold in RH-35 cells incubated 5 hours with 2 μM hydrocortisone 21-succinate as compared with activities of control cells incubated 5 hours in the absence of steroid. The time course and extent of induction of TAT by dexamethasone in RH-35 cells are in general agreement with the findings of others (21).

Lysosomal enzyme activity of nuclei during induction of TAT. In order to determine if glucocorticoids stimulated an increase in liver nuclear lysosomal enzyme activity in a manner similar to that reported by

Figure 1. Growth Curve for RH-35 Cells. 3.4×10^5 cells were innoculated into tissue culture flasks containing 15 ml MEM with 10% FBS and incubated as described in Materials and Methods section. Medium change is indicated by arrows. Each point is the mean ± S.D. for three or four flasks.

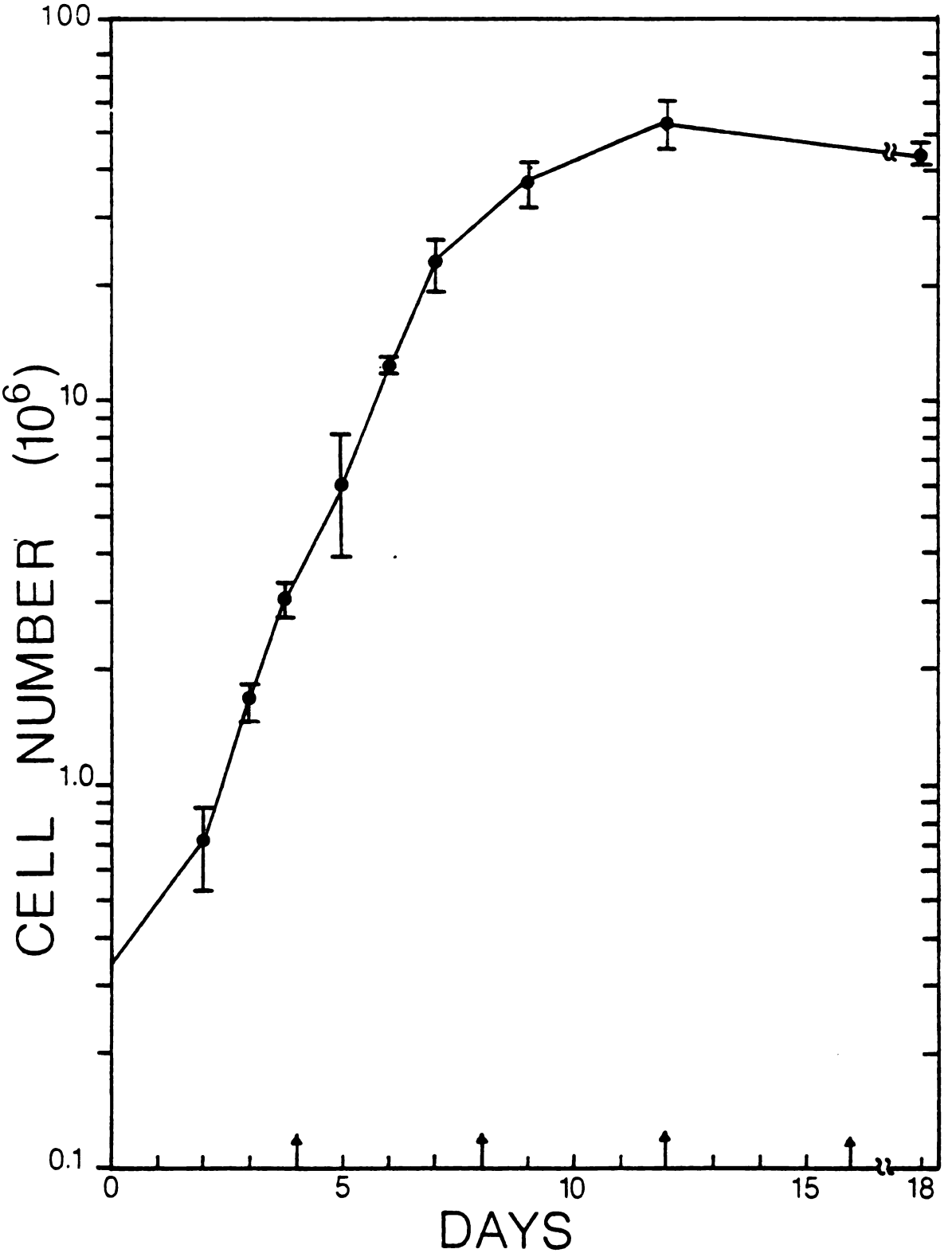


Figure 1. Growth Curve for RH-35 Cells.

Figure 2. Effect of Dexamethasone Concentration on Tyrosine Aminotransferase Activity. RH-35 cells grown to confluency were incubated in FBS-deficient medium 19 hours prior to initiation of induction. At 0 hour, 1 ml of concentrated stock solutions of dexamethasone were added to the incubation medium resulting in the final concentrations indicated. Cells were incubated 12 hours with dexamethasone then harvested by trypsinization, fractionated, and analyzed as described in Materials and Methods. Values are means ± S.D. for three flasks.

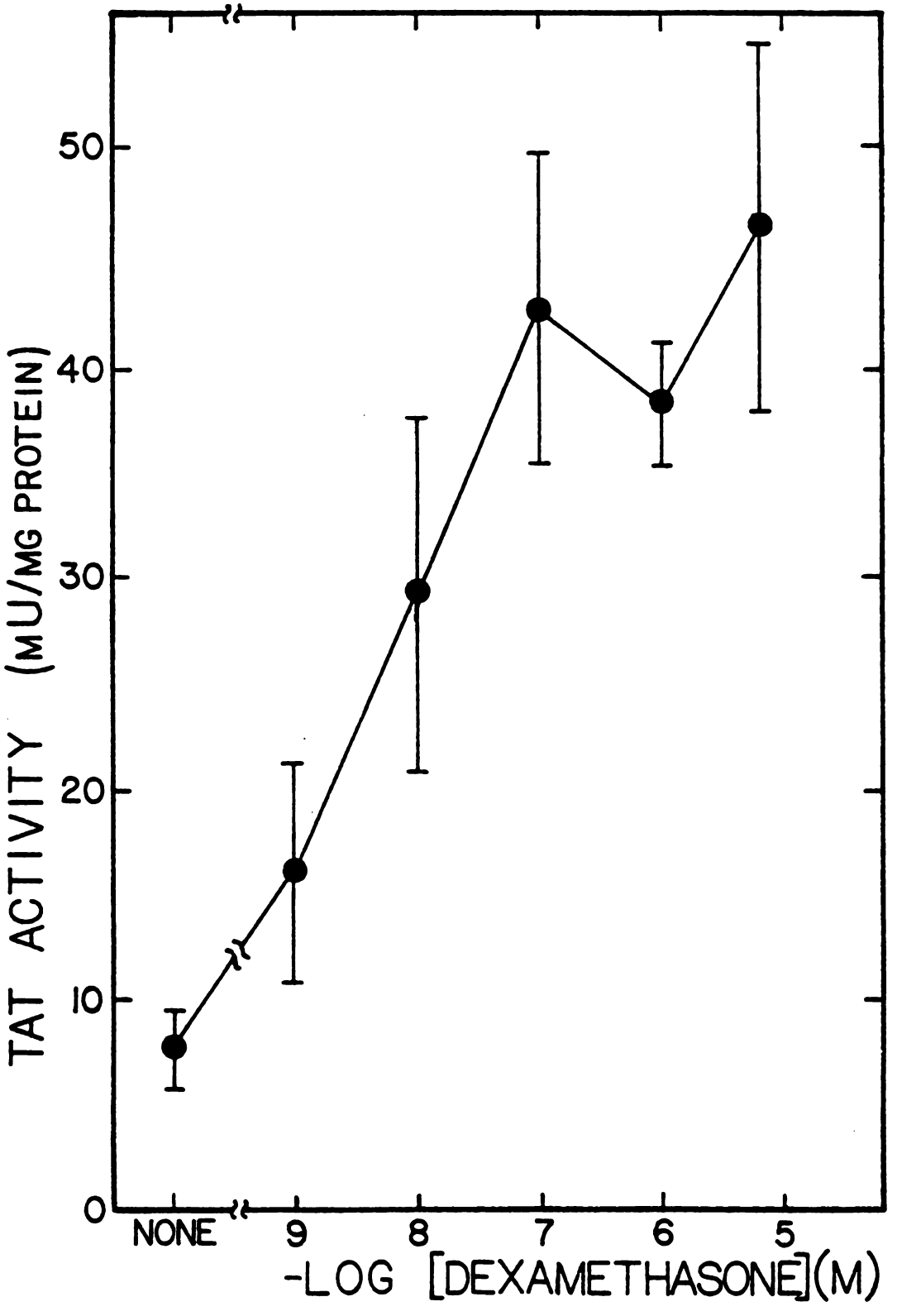


Figure 2. Effect of Dexamethasone Concentration on Tyrosine Aminotransferase Activity.

Figure 3. Time Course of Induction of Tyrosine Aminotransferase in RH-35 Cells by Dexamethasone. Conditions were as described in Figure 2. Dexamethasone concentration was 1 μM. Values are means ± S.D. for three flasks.

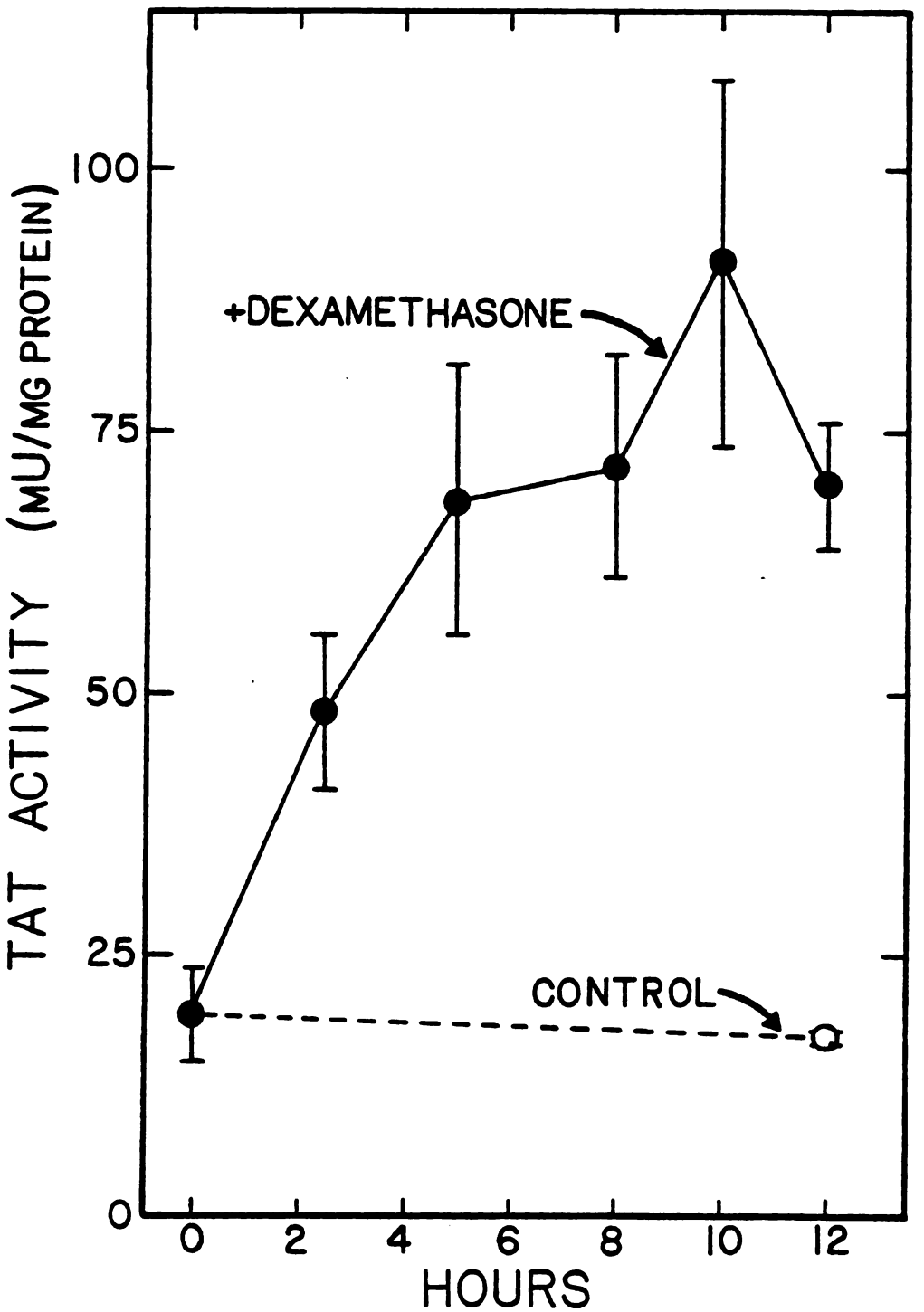


Figure 3. Time Course of Induction of Tyrosine Aminotransferase in RH-35 Cells by Dexamethasone.

Szego et al. (2) for estradiol action in rat uterus and preputial glands, the following experiments were performed. Figure 4A shows the time course of nuclear hexosaminidase activity in RH-35 cells incubated in the presence and absence of 2 μM dexamethasone. Nuclear hexosaminidase activity of control and dexamethasone treated cells did not differ except at the 3.5 hour time point where control nuclear activity was nearly twice that of dexamethasone treated cells. Hexosaminidase activities determined in the absence of 0.2% triton X-100 were 50-70% of the activity in the presence of triton X-100 and paralleled activities determined with detergent. This constant amount of structural latency is consistent with the presence of lysosomes in the preparations of nuclei. TAT activity of the dexamethasone treated cells increased 4 to 5-fold during this time course (Figure 4B). In this experiment the induction was begun by a change of medium. In order to minimize non-hormone effect of medium change, in the experiment shown in Figure 5, a small amount of concentrated dexamethasone was added to cultures without changing the medium. Also, earlier time points were examined. Nuclear β-glucuronidase, cathepsin D, and hexosaminidase activities changed in parallel during the course of incubation of RH-35 cells with 1 μM dexamethasone. This observation is consistent with the presence of a single class of lysosomes in nuclear fractions. Hexosaminidase activity of nuclear fractions, but not that of β-glucuronidase or cathepsin D, was significantly elevated at 15 min compared with 0 min. However this increase proved to be irreproducible.

In order to determine if the association of lysosomes with nuclei was a loose one which was perhaps disrupted by homogenization and purification of nuclei, RH-35 cells were examined microscopically after the

Figure 4. Time Course of Tyrosine Aminotransferase and Nuclear Hexosaminidase Activity in RH-35 Cells Incubated with Dexamethasone. Confluent cultures of RH-35 cells were incubated in FBS-deficient EMEM for 19 hours prior to the start of the experiment. At 0 time, medium was changed to FBS-deficient EMEM with (circle) and without (squares) added dexamethasone at a concentration of 2 x 10 μM. At the times indicated, cells were harvested by trypsinization then fractionated and analyzed as described in Materials and Methods. Purified nuclei (panel A) were assayed for hexosaminidase activity with (closed symbols) and without (open symbols) a final concentration of 0.2% triton X-100. TAT activity (panel B) was determined in 22,000 x g supernatants as described in Materials and Methods. Values are means ± S.D. for three flasks.

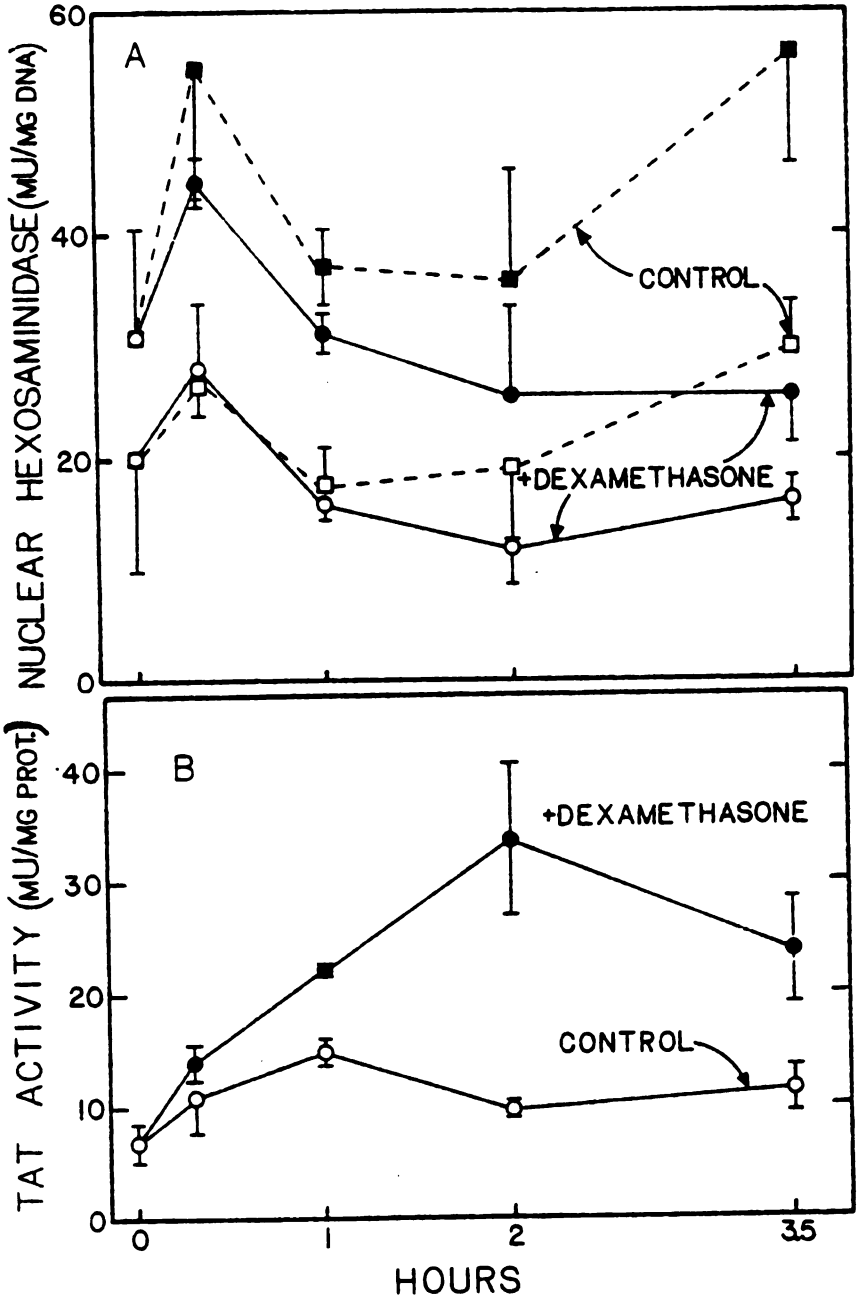


Figure 4. Time Course of Tyrosine Aminotransferase and Nuclear Hexosaminidase Activity in RH-35 Cells Incubated with Dexamethasone.

Figure 5. Lysosomal Enzyme Activity of Nuclei from RH-35 Cells Incubated with Dexamethasone. Confluent cultures of RH-35 cells were incubated in 10 ml FBS-deficient MEM 12 hours prior to the start of the experiment. Experiment was begun by the addition of concentrated stock dexamethasone to a final concentration of 1 μM. At the times indicated, cells were harvested with a rubber policeman in 4.5 ml 0.32 M sucrose, 3 mM $MgCl_2$ and fractionated as described in the Methods section. Hexosaminidase and β-glucuronidase activities are mU/mg DNA and cathepsin D activity is cpm [^{3}H]-peptides released/min/mg DNA. Values are means ± S.D. for four T-flasks.

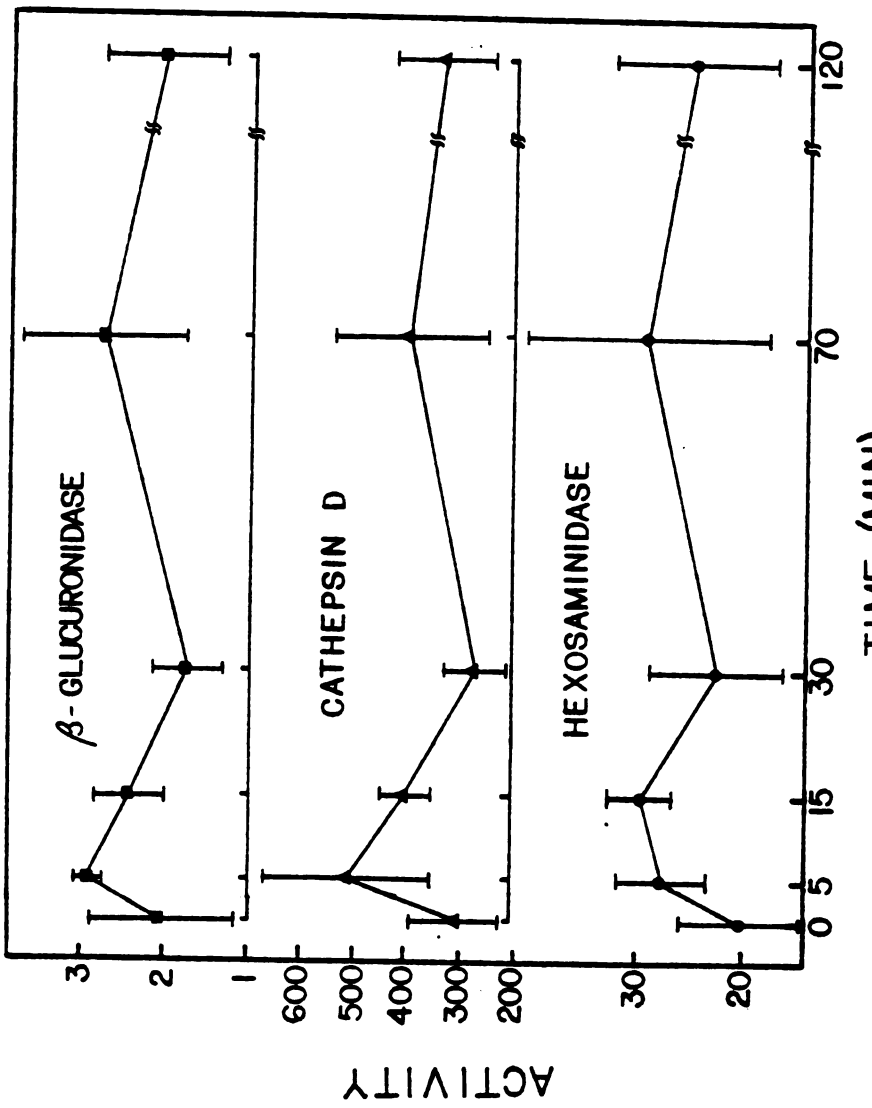


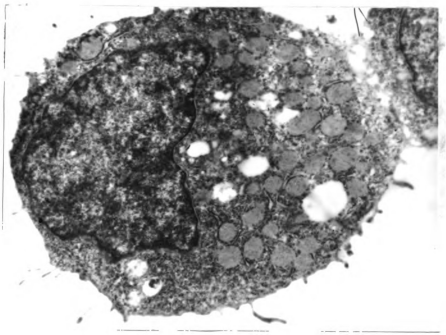
Figure 5. Lysosomal Enzyme Activity of Nuclei from RH-35 Cells Incubated with Dexamethasone.

addition of 1 μM dexamethasone. At various times after hormone additions, cells were fixed, stained for lysosomes by the Gomori's lead medium (11), then further prepared and examined under the electron microscope. The results of this experiment are shown in Figure 6A-F. Lysosomes are identifiable by the halo of electron opaque lead phosphate which precipitates around the lysosome as the result of a reaction of lead in the medium with the phosphate liberated from β-glycerophosphate by the action of acid phosphatase. Golgi and endoplasmic reticulum are known to stain by this method (11) as is evident in Figure 6. There was no evidence for a fusion or peri-nuclear localization of lysosomes in the cells examined. Figure 6E, 10 min after exposure of cells to 1 μM dexamethasone, showed a close association of lysosomes with nuclei but this was not a common observation.

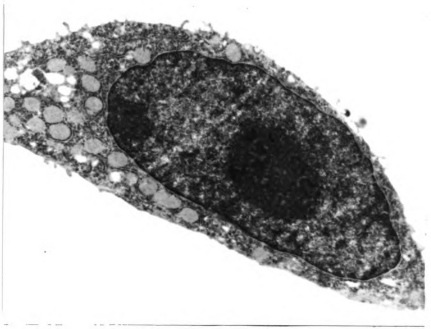
In order to determine whether changes in nuclear lysosomal enzyme activities were small in relation to endogenous activity and therefore difficult to demonstrate, nuclear hexosaminidase and acid phosphatase activities were determined in normal and RH-35 cells. Results shown in Table I indicated that RH-35 cells had 3-times more hexosaminidase activity per mg nuclear DNA than normal hepatocytes. As a percentage of total cellular hexosaminidase, RH-35 cell nuclei had 9-fold more hexosaminidase activity than present in hepatocyte nuclei. Preliminary examination of acid phosphatase activities indicates the reverse pattern of association.

In order to examine the possibility that glucocorticoids <u>in vivo</u> might therefore stimulate an observable increase in nuclear lysosomal enzyme activity in a manner similar to estradiol action reported by Szego, the following experiment was performed. Rats were

Figure 6. Electron Microscopic Examination of Intracellular Lysosome Distribution after Treatment of RH-35 Cells with Dexamethasone. Confluent cultures of RH-35 cells containing FBS-deficient MEM were exposed to dexamethasone (1 μM). At (A) 0 time, (B) 0.5 min, (C) 2 min, (D) 4 min, (E) 10 min, and (F) 30 min, medium was decanted and cells were fixed for 20 min with 2% glutaraldehyde then stained 40 min with Gomori acid phosphatase stain. Cells were then dehydrated with ethanol, embedded in Epon-Araldite, thin sectioned and stained with saturated uranyl acetate then post-stained in saturated lead citrate. Magnifications are 10,000 and 40,000.

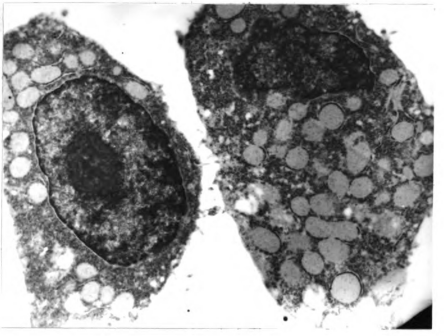


A 0 minutes, 10,000X

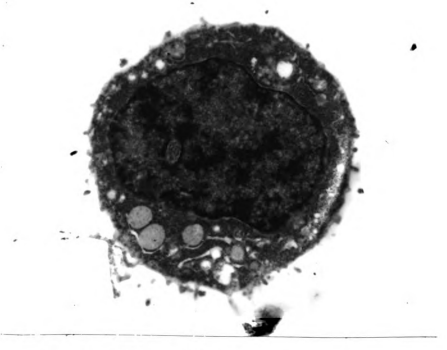


B 0.5 minutes, 10,000X

Figure 6 A and B. Electron Microscopic Examination of Intracellular Lysosome Distribution after Treatment of RH-35 Cells With Dexamethasone.

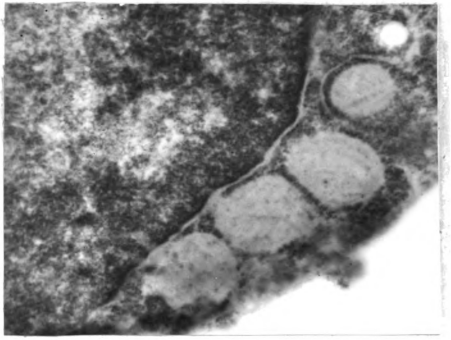


C 2 minutes, 10,000X

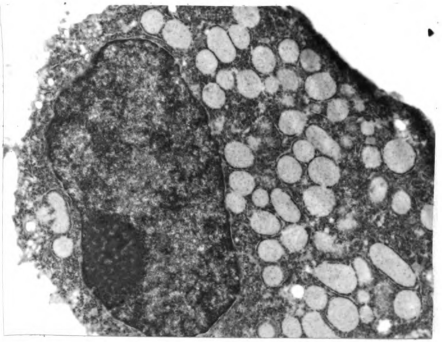


D 4 minutes, 10,000X

Figure 6 C and D. Electron Microscopic Examination of Intracellular Lysosome Distribution after Treatment of RH-35 Cells With Dexamethasone.



E 10 minutes, 40,000X



F 30 minutes, 10,000X

Figure 6 E and F. Electron Microscopic Examination of Intracellular Lysosome Distribution after Treatment of RH-35 Cells With Dexamethasone.

Table I. Enzyme Activities of Rat Hepatocyte and RH-35 Cell Fractions.[1]

Fraction	Enzyme	Activity	
		Rat hepatocytes	RH-35 cells
WH	hexosaminidase	408.6 ± 55.8	139.0 ± 59.0
	acid phosphatase	66.1 ± 2.3	9.20 ± 4.40
PN	hexosaminidase	6.31 ± 3.68	19.2 ± 14.7
	acid phosphatase	4.71 ± 0.82	2.99 ± 1.08
(PN/WH)100	hexosaminidase	1.39 ± 0.72	12.6 ± 5.9
	acid phosphatase	7.2 ± 1.4	41 ± 26

[1]Whole homogenate (WH) and purified nuclear (PN) fractions were prepared as described under Methods. Hexosaminidase activity is mU 4-MU/mg DNA. Rat hepatocyte values are from 3 rats with 11 observations and RH-35 cell values are from 2 different passages of cells with 5 observations. Acid phosphatase activity is μmoles Pi/hr/mg DNA. Rat hepatocyte values are from 1 rat with 3 observations and from 1 passage of RH-35 cells with 3 observations. Rat hepatocyte values are from 4 rats with 14 observations and RH-35 cell values are from 3 different passage of cells with 8 ovservations. Values are means ± S.D. with 4×10^7 cells per observation.

adrenalectomized and maintained for five days to remove endogenous glucocorticoids. Rats were then starved for 18 hours in order to put them in a physiological state which would require glucocorticoids. Animals then received 10 mg/100 g body weight hydrocortisone 21-succinate and were sacrificed at various times after injection. This dose of hydrocortisone is optimal for the *in vivo* induction of TAT in adrenalectomized rats (14). Purified liver nuclei were isolated and analyzed for lysososmal enzyme activity at various times after injection. The results of two such experiments are shown in Figure 7A and 7B. In one experiment (Figure 7A) nuclear cathepsin D and acid phosphatase activities declined steadily after injection of hydrocortisone to 60 and 40% respectively of initial activities at 45 min after injection. In a second experiment (Figure 7B) it was noted that adrenalectomy alone lowered the nuclear acid phosphatase activity to values 50% of those in unoperated or sham operated rat liver nuclei. Injection of hydrocortisone into adrenalectomized rats had no effect on nuclear acid phosphatase during the early period of TAT induction. Injection of unoperated or sham operated rats with saline decreased nuclear acid phosphatase activity to values 70% of initial activities at 60 min after injection. Saline injection into adrenalectomized rats slightly increased nuclear acid phosphatase activity by 60 min after injection.

Figure 7. Lysosomal Enzyme Activity of Liver Nuclei from Adrenalectomized Rats after Injection of Hydrocortisone 21-Sodium Succinate. Panels A and B are two separate experiments of the same design. Male rats (130-195 g) were adrenalectomized or sham operated, maintained for 5 days, then starved 18 h prior to injection with 10 mg hydrocortisone 21-sodium succinate/100 g body weight or saline. Animals were sacrificed at timed intervals after injection and livers were processed as described in Materials and Methods to obtain purified nuclear fractions for lysosomal enzyme activity analysis. Symbols: unoperated, uninjected; unoperated, saline injected; sham operated, uninjected; sham operated, saline injected; adrenalectomized, uninjected; adrenalectomized, saline injected; adrenalectomized, hydrocortisone injected. Values are means ± S.D. for 3 rats per point.

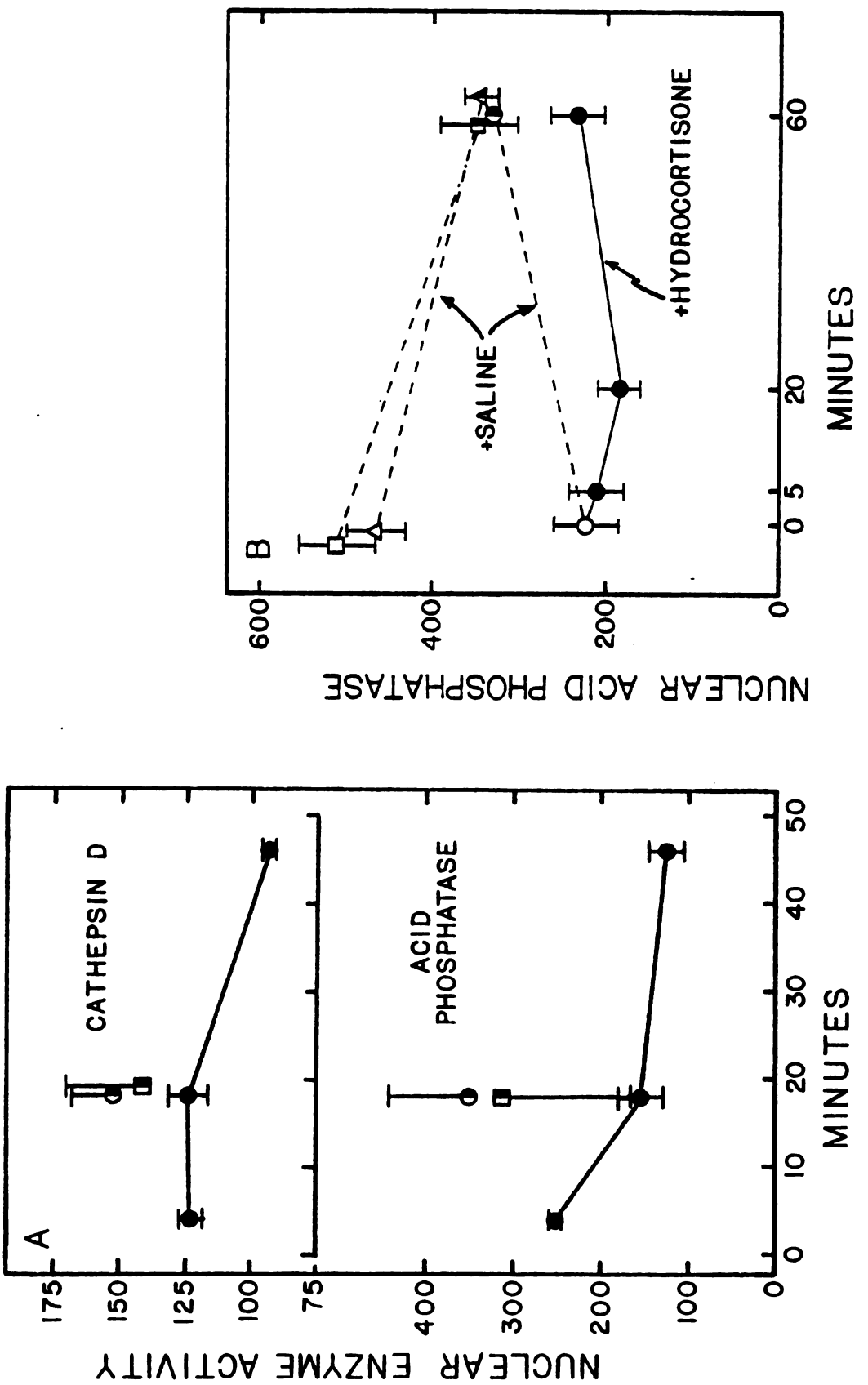


Figure 7. Lysosomal Enzyme Activity of Liver Nuclei from Adrenalectomized Rats after Injection of Hydrocortisone 21-Sodium Succinate.

DISCUSSION

Following diffusion into the cell (15, 16), glucocorticoids bind to glucocorticoid binding protein G (17) which is involved in TAT induction by stimulating RNA synthesis (18, 19). The mechanism by which the glucocorticoid-receptor G complex moves to the nucleus is unknown. Most authors suggest that translocation of steroid-receptor complexes from the cytoplasm into the nucleus is dependent upon the binding of steroid-receptor complexes to high affinity binding sites of chromatin (20). Movement to the nucleus would occur by diffusion in order to maintain a homogenous intracellular distribution of free receptor-hormone complexes. Gannon et al. (20) suggest that the hormone-receptor complex is "excluded" from the cytoplasm and preferentially accumulated in the nucleus due to the high intranuclear water content (1.6:1 = nuclear H_2O:cytosol H_2O). Szego has evidence (2) that lysosomes move to and associate with nuclei in response to estradiol stimulation of target cells and that in this way the estradiol-receptor complex is transported into the nucleus.

Of primary importance is the demonstration that the RH-35 cells in culture respond in a typical fashion to added glucocorticoids. The results presented in Figures 1, 2, and 3 confirm the well documented growth characteristics and inducibility of TAT in RH-35 cells in culture in response to added dexamethasone (21). The value of total rat hepatocyte acid phosphatase reported in Table I (66.1 ± 2.3 μmoles Pi/hr/mg DNA) is in agreement with that reported by Munthe-Kaas et al. (22) (approximately 100 μmoles Pi/hr/mg DNA). The hexosaminidase value reported in Table I (408.6 ± 55.8 nmoles 4 MU/min/mg DNA) is lower

than that reported by Munthe-Kaas et al. (approximately 900 nmoles p-nitrophenol/min/mg DNA) (22). This discrepancy could be explained by differences in temperature of assay (37°C for Munthe-Kaas et al. (22) versus 25°C for results presented here) as well as the known variations of hexosaminidase activity towards different substrates (23-25). The lysosomal enzyme activities of RH-35 cells and nuclear fractions of normal hepatocytes have not been previously reported.

The remaining experiments conducted here (Figures 4 to 7) were performed to examine the possibility that the translocation concept of Szego, proposed originally for estradiol action (2), could be extended to include glucocorticoids. This seemed likely because of the many similarities of the early events of estradiol (26) and glucocorticoid (1, 27, 28) action i.e., steroid hormones, cytosol receptors of 200,000 MW, temperature dependent conformational change of receptor, translocation to the nucleus, binding to DNA, and specific stimulations of transcription.

The biochemical and microscopic results presented here (Figures 4-7) do not support the extension of Szego's model for estradiol action to include glucocorticoid action in RH-35 cells or rat liver. Since the method of nuclei preparation was according to Szego and Seeler (3) and the enzyme analyses used were sufficiently sensitive to detect changes of the magnitude reported (10-15 fold) in "purified nuclei" of preputial glands stimulated with estradiol (4) it is difficult to suggest that such a phenomenon occurred but was not detected. An alternate possibility is that liver lysosomes do move to the nuclei in response to glucocorticoid stimulation but not to the same extent as observed in preputial glands or uteri under the influence of estradiol. Examination of this possibility

would require the development of more sensitive techniques. Of interest is the finding (Table I) that nuclei of RH-35 cells have higher activities of hexosaminidase than normal rat hepatocytes despite lower activity per total cellular DNA. A preliminary comparison of RH-35 and normal rat hepatocyte nuclear acid phosphatase activities revealed the opposite trend (Table I). An intriguing possibility is that this is a general characteristic of tumor cells.

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Chapter III

Induction of Lipogenesis Enzymes in Rat Liver During Starvation-Refeeding. Investigation of the Involvement of Lysosomes.

ABSTRACT

The intracellular distribution of lysosomal enzymes was examined during the course of G6PDH and 6PGDH induction by a regimen of starvation/refeeding. Lysosomal fragility was determined by the non-particulate activity as a percentage of total activity (i.e., percent free activity) under standard homogenization procedures. For acid phosphatase, hexosaminidase, and β-galactosidase, percentage free activity increased significantly (42, 120, and 42% respectively) in comparison with normal rat liver levels at 9 hours after feeding a high carbohydrate diet to 3 day starved rats. Mitochondrial fragility, as measured by the percentage free fumarase activity, was not significantly altered from a value of approximately 50% during the course of starvation/refeeding. Labilization or stabilization of lysosomes by a single glucose injection (750 mg/100 g body weight) into fed rats or corticosterone injection (2.5 mg/100 g body weight) during a starvation/refeeding regimen, respectively, was not correlated with changes in the capacity for induction of G6PDH and 6PGDH when compared with respective controls. Nuclear hexosaminidase specific activity but not that of cathepsin D or acid phosphatase, increased significantly above normal levels during a 3 day starvation period. Refeeding of a high glucose diet to 3 day starved rats resulted in no significant alteration in the association of lysosomal

enzyme activities with purified nuclei compared with activities present in nuclei from 0 hour refed rats. Starved/refed streptozotocin diabetic rats, with or without insulin injection at doses sufficient to induce G6PDH and 6PGDH, showed no significant change in purified nuclear associated acid phosphatase activity throughout the experiment. The results presented here do not support the role for lysosomes as a mediator of enzyme induction in the starved-refed rat as suggested by Szego (see references 1 and 2) for estradiol and ACTH action.

INTRODUCTION

Starvation followed by refeeding results in elevations of liver lipogenic enzymes above normal levels (3). The mechanism by which diet and insulin (see Chapter 4) serve to induce G6PDH and 6PGDH is not clear. Szego et al. (for review see 1 and 2) have presented evidence that, in estradiol responsive systems, estradiol administration resulted in the rapid (within 2 min) association of lysosomes with nuclei (4,5) as well as lysosome fragility (4,6). Previous work from this laboratory (7,8) has indicated that the starvation-refeeding stimulated induction of G6PDH and 6PGDH was associated with lysosome changes observed by Szego et al. in estradiol responsive tissues. The purpose of the present investigation was to further examine the involvement of lysosomes, both fragility and nuclear association, in the process of dietary and hormonal stimulation of G6PDH and 6PGDH induction.

MATERIALS AND METHODS

Animal treatment. Male rats weighing initially 150-200 g were purchased from the Holtzman Co., Madison, WI and housed and fed rat chow (Wayne Lab-Blox, Allied Mills, Inc.) as described (7). The high glucose diet was 68.9% glucose, 20% casein, 5% corn oil, 5% salt mix (Wesson, Osborn modified), 1% vitamin mix (Nutritional Biochemicals Inc.) and 0.1% choline chloride. The Wesson (Osborn modified) salt mix was supplemented to 10 ppm Cu^{2+} using $CuSO_4$, 50 ppm Mn^{2+} using $MnSO_4$, and 50 ppm Zn^{2+} using ZnO as recommended by Greenfield and Briggs (9). Streptozotocin, a gift from Dr. W.E. Dulin of Upjohn Co., Kalamazoo, MI, was dissolved in 0.1 M sodium citrate buffer, pH 4.5 and injected into the femoral vein of ether anesthetized rats within 10 min of solution preparation. Hepatocytes were isolated from rats by the collagenase method of Seglen (10) as described in detail in the Materials and Methods section of Chapter 4 of this dissertation. Insulin, Regular and Lente Iletin, 100 U/ml, was from Eli Lilly and Co.

Sample preparation. Rats were sacrificed by decapitation and livers quickly removed and placed in 0.25 M sucrose, 2 mM $MgCl_2$. Homogenization and fractionation by differential centrifugation were as described previously (7) to obtain 800 x g - 15 min and 22,000 x g - 15 min supernatant fractions for analysis of lysosomal enzyme activities and a 105,000 x g - 60 min fraction for analysis of dehydrogenase activity. Hepatocytes were fractionated as described previously (11). Purified nuclei were obtained using the procedure of Szego and Seeler (4) except high-speed centrifugation was with a fixed-angle rotor (30K) for liver samples and a swinging bucket rotor (SW 27.1) for isolated hepatocytes.

Enzyme analyses. Acid phosphatase activity was determined at 25°C, pH 5.0 by the rate of release of phosphate from β-glycerophosphate by two different methods. Method 1 was described by Schroeder et al. (7) and method 2 was described by Mak and Wells (8). Both methods have the same initial assay conditions which are a modification of those described by Vaes and Jacques (12). The methods differ in the detection of phosphate. Method 1 utilizes an isobutanol:benzene extraction of TCA soluble phosphate and method 2 measures free phosphate in the reaction mixture after clarification of samples with sodium dodecyl sulfate. Hexosaminidase activity was determined at 37°C, pH 3.8 by the rate of release of p-nitrophenol from p-nitrophenyl-N-acetyl-β-D-glucosaminide as described by Schroeder et al. (7). β-Galactosidase activity was determined at 37°C, pH 5.0 by the rate of release of p-nitrophenol from p-nitrophenyl-β-D-galactopyranoside as described by Schroeder et al. (7) except that the final substrate concentration was raised to 1.5 mM. β-Glucuronidase activity was determined at 37°C, pH 5.0 by the rate of release of p-nitrophenol from p-nitrophenyl-β-D-glucuronide as described by Schroeder et al. (7). Cathepsin D activity (method 1) was determined at 45°C, pH 3.4 by the rate of release of TCA soluble peptides as tyrosine equivalents from hemoglobin substrate as described by Barrett (13) or (method 2) by the rate of release of TCA soluble [^{3}H]-acetyl-peptides from [^{3}H]-acetyl-hemoglobin at pH 3.0, 45°C as described by Barrett (13). Method 2 is described in detail in the Materials and Methods section of Chapter 2 of this dissertation. Tyrosine equivalents were determined by the method of Lowry et al. (14) using L-tyrosine as a standard. All lysosomal enzyme activities were determined in the presence of 0.2% (w/v) Triton X-100 (Rohm and Haas). Fumarase activity was determined at

25°C, pH 7.4 by the increase in C-C double bond formation from L-malate as described by Hill and Bradshaw (15). G6PDH and 6PGDH activities were determined by the spectrophotometric method described previously (11). Lactate dehydrogenase activities were determined at 25°C by measuring the rate of reduction of NAD^+ in the presence of L-lactate using the lactate dehydrogenase kit (LD-P14, Gilford Diagnostics) in conjunction with a Gilford 3500 spectrophotometer. Protein was determined according to Lowry et al. (14) with bovine serum albumin as the standard and DNA was determined by the diphenylamine method of Giles and Myers (16) as described previously (11). All biochemicals were purchased from Sigma Chemical Co., St. Louis, MO except where indicated otherwise. Statistical methods were according to Steel and Torrie (17) with $\alpha = 0.05$. Means not having a common superscript are significatly different as determined by analysis of variance, least significant difference test.

RESULTS

G6PDH and 6PGDH induction and liver lysosome fragility. Figure 1A and 1B shows the effects of hydrocortisone injection on the fragility of rat liver lysosomes and mitochondria during a starvation/refeeding regimen. The percentage free activity i.e., the activity/ml of the post-mitochondrial-lysosomal supernatant (22,000 x g - 15 min) as a percent of the activity/ml of the post nuclear supernatant (800 x g - 15 min), is used as an index of lysosome fragility. The percent free activity of liver acid phosphatase, hexosaminidase, and β-galactosidase of normal control rats were 17.2 ± 2.1, 4.1 ± 1.0, and 11.8 ± 1.6% respectively. Feeding rats a high glucose diet for 8 days led to a slight but not significant elevation in the percentage free activity of acid phosphatase, hexosaminidase, and β-galactosidase compared with the percentage free activity of these enzymes in livers of rats fed a chow diet (Figure 1A and 1B). Starvation for 3 days (0 hour refed) led to a significant increase only in percentage free hexosaminidase activity compared with percent free hexosaminidase activities in livers of rats fed a chow diet. Refeeding 3 day starved rats a high glucose diet for 9 hours resulted in significant elevations in the percent free activity of all three liver lysosomal enzymes above the respective percent free activities present in livers of chow diet fed control rats. The percent free activities of acid phosphatase, hexosaminidase, and β-galactosidase were 24.4 ± 2.4, 9.0 ± 2.7, and 16.7 ± 1.9, respectively. This peak in percent free activity was followed, after 2 days of refeeding, by a return to normal levels of percent free activity. The percent fumarase which was free in normal chow fed rats was 49.9 ± 6.2%. Starvation-refeeding had no significant

Figure 1. Effect of Hydrocortisone Injection on Liver Lysosome and Mitochondria Fragility of Starved-Refed Rats. Male Holtzman rats weighting initially 200-230 g were used. Controls were fed rat chow (C) or the high glucose diet (G) (see Materials and Methods) for 8 days. "Refed" animals were starved for 3 days, refed the high glucose diet at 0 hour, and sacrificed at the times indicated. Livers were prepared and analyzed as described in Materials and Methods. "Percent free" activity is the activity/ml of the post-lysosomal (22,000 x g - 15 min) supernatant as a percent of the activity/ml of the post-nuclear (800 x g - 15 min) supernatant. Percent free activities of acid phosphatase (analyzed by method 1) and hexosaminidase are shown in panel A and β-galactosidase and fumarase are shown in panel B. Open circles indicate rats were uninjected and closed circles indicate rats were injected with 2.5 mg hydrocortisone/100 g body weight. Injection times were -0.5, 0.5, 5, 7, and 9 hours. Values are ± S.D. of 4 rats.

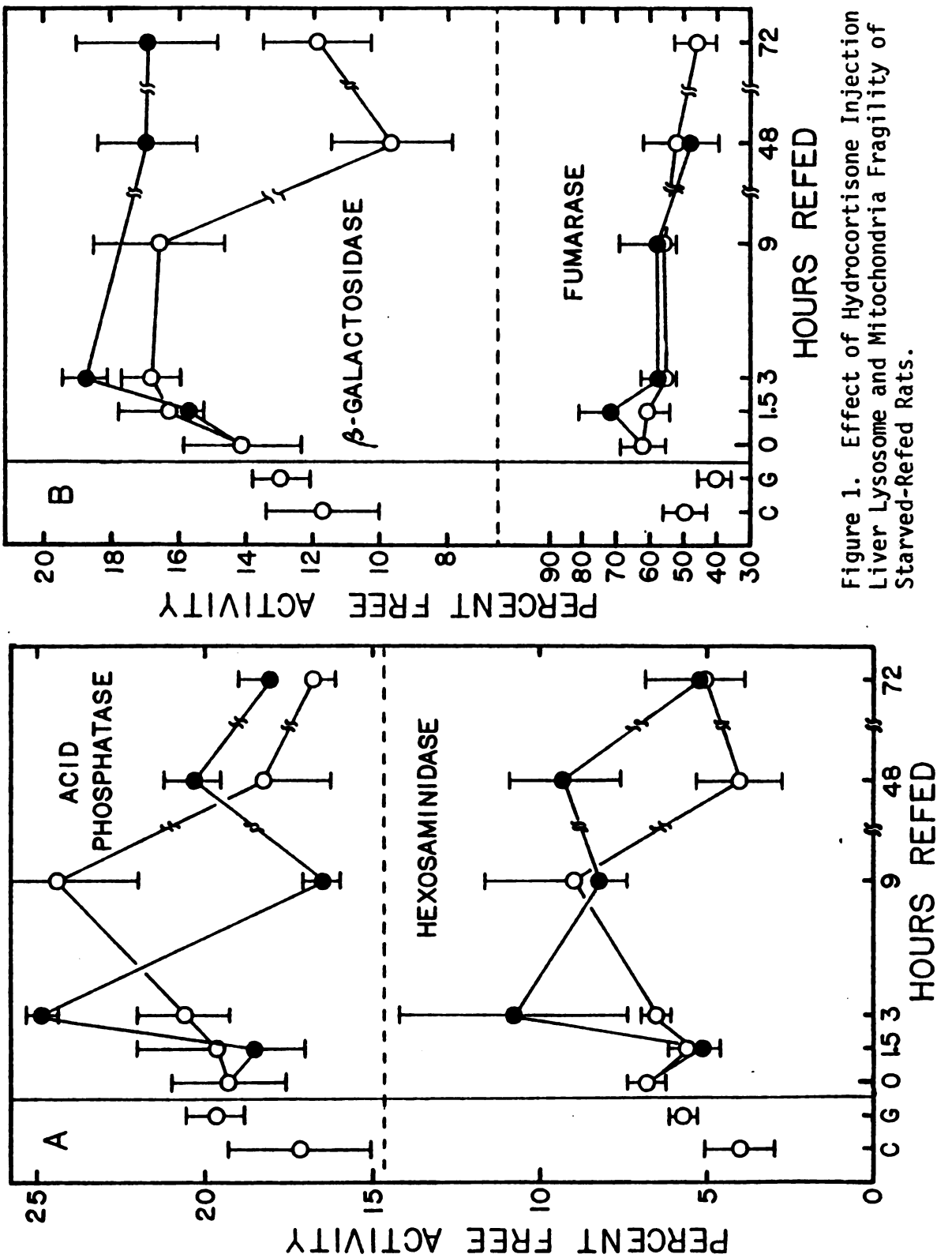


Figure 1. Effect of Hydrocortisone Injection on Liver Lysosome and Mitochondria Fragility of Starved-Refed Rats.

effect on the percent free fumarase activity (Figure 1B). Hydrocortisone injection during starvation/refeeding resulted in significant increases, at 3 hours after refeeding, in the percentage free activity of acid phosphatase, hexosaminidase, and β-galactosidase above respective liver percent free activities of 0 hour refed and chow diet fed rats. Injection of hydrocortisone appeared to shift the transient peak in lysosomal fragility from 9 hours to 3 hours after refeeding the high glucose diet. The percent free fumarase activity also was elevated by hydrocortisone injection to activities above free fumarase activities of control rats fed a chow diet.

G6PDH and 6PGDH activities were examined in this experiment; the results are shown in Figure 2. Induction of these enzymes occurred after 2 days of refeeding the high glucose diet. Injection of hydrocortisone resulted in a significant impairment in the induction of G6PDH but not 6PGDH activity compared with the induction of liver G6PDH and 6PGDH activity of starved, 2 day refed uninjected rats. This difference between liver G6PDH activities of control and hydrocortisone injected rats was not present at 3 days of refeeding however. Liver and body weights for this experiment are shown in Table I.

In an experiment similar to the one just described, the effects of corticosterone injection on liver lysosome fragility and the induction of G6PDH and 6PGDH were examined. Results of this experiment are shown in Tables II and III. Compared with normal chow fed rats, starvation had no effect on the percent free hexosaminidase or β-galactosidase activity. Refeeding of 3 day starved rats with the high glucose diet for 6 hours resulted in a significant 2.7-fold increase in the percent free

Figure 2. Effect of Hydrocortisone Injection on Liver Glucose 6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase Activities of Starved-Refed Rats. This is a continuation of the experiment described in the legend to Figure 1. Dehydrogenase activities were performed on 105,000 x g - 60 min supernatants. Symbols: (○) G6PDH and (□) 6PGDH. Open symbols indicate rats were uninjected and closed symbols indicate rats were injected with 2.5 mg hydrocortisone/100 g body weight. C and G are chow and high glucose diet fed respectively for 8 days. Values are means ± S.D. of 4 rats.

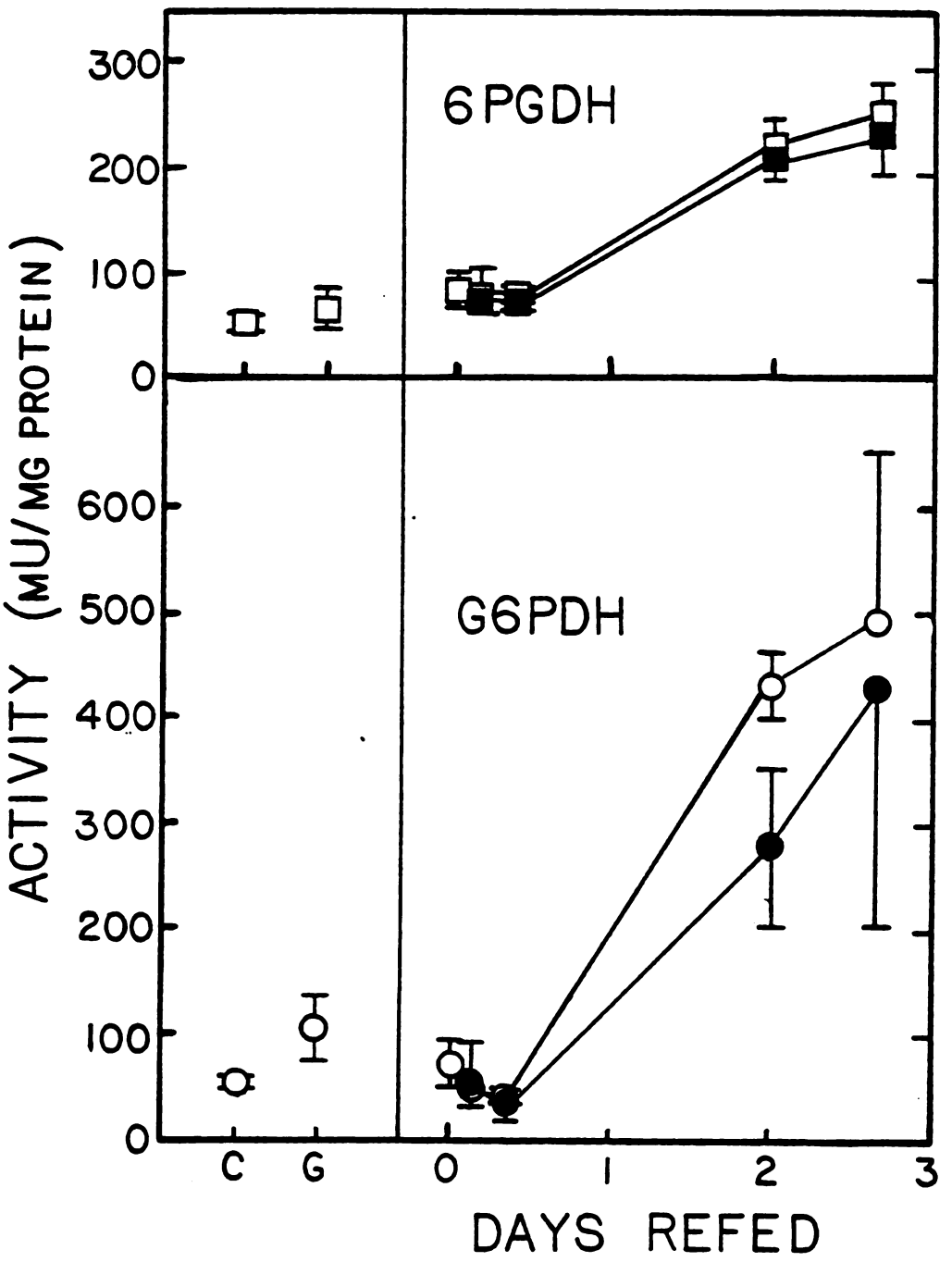


Figure 2. Effect of Hydrocortisone Injection on Liver Glucose 6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase Activities of Starved-Refed Rats.

Table I. Liver and Body Weights of Normal and Hydrocortisone Injected Rats During Starvation-Refeeding.[a]

Treatment	Final Body Weight (g)		$\frac{\text{Liver Weight}}{\text{Body Weight}}$ (%)	
	hydrocortisone injected[b]		hydrocortisone injected[b]	
	-	+	-	+
fed rat chow	287 ± 9	-	4.88 ± 0.18	-
fed glucose diet	272 ± 8	-	4.60 ± 0.14	-
starved 3d	211 ± 11	-	3.29 ± 0.18	-
starved 3d, refed 1.5h	219 ± 6	208 ± 13	3.42 ± 0.14	3.26 ± 0.22
starved 3d, refed 3h	221 ± 7	221 ± 10	3.26 ± 0.23	3.38 ± 0.20
starved 3d, refed 9h	219 ± 7	216 ± 5	3.74 ± 0.12	4.19 ± 0.17
starved 3d, refed 48h	243 ± 7	222 ± 11	5.42 ± 0.12	5.53 ± 0.39
starved 3d, refed 72h	233 ± 3	238 ± 9	5.23 ± 0.26	5.23 ± 0.33

[a]This is a continuation of the experiment described in the legend to Figure 1. Values are means ± S.D. of 4 rats.

[b]Dosage of 2.5 mg/100 g body weight.

Table II. Effect of Corticosterone Injection on Liver Lysosome Fragility in Starved-Refed Rats.

	Hexosaminidase (% free)		β-Galactosidase (% free)	
	cortisosterone injected		corticosterone injected	
Treatment	-	+	-	+
fed rat chow	4.4 ± 1.2[a,b]	-	12.4 ± 2.6[a,b]	-
fed glucose diet	6.2 ± 1.8[b]	-	21.4 ± 2.4[b]	-
starved 3d	5.8 ± 1.4[a,b]	-	13.3 ± 2.2[a,b]	-
starved 3d, refed 3h	3.6 ± 0.8[a,b]	5.0 ± 1.1[a,b]	32.8 ± 11.0[b,c]	15.4 ± 8.2[a,b]
starved 3d, refed 6h	3.9 ± 0.1[a,b]	4.0 ± 0.7[a,b]	36.0 ± 1.5[c]	12.5 ± 2.9[a,b]
starved 3d, refed 48h	4.0 ± 1.1[a,b]	3.4 ± 1.1[a]	10.5 ± 5.3[a,b]	6.8 ± 3.2[a]

[a]Male Holtzman rats weighing initially 150-200 g were used. Controls were fed rat chow or the high glucose diet (see Materials and Methods) for 11 days. "Refed" animals were starved for 3 days, refed the high glucose diet at 0 hour, and sacrificed at the times indicated. Livers were prepared as described in Materials and Methods. Percent free activity is the activity/ml of the post-lysosomal (22,000 x g-15 min) supernatant as a percent of the activity/ml of the post-nuclear (800 x g-15 min) supernatant. The + and - signs refer to presence or absence of injection with 2.5 mg corticosterone/100 g body weight. Injection times were -1.5, 1.5, and 4.5 hours relative to refeeding at 0 hour. Values are means ± S.D. of 3 rats.

Table III. Effect of Corticosterone Injection on Liver G6PDH and 6PGDH in Starved-Refed Rats.[a]

	G6PDH		6PGDH	
	corticosterone injected[b]		corticosterone injected[b]	
Treatment	-	+	-	+
fed rat chow	29.0 ± 6.6	-	99.0 ± 19.7	-
fed glucose diet	149.5 ± 2.4	-	147.4 ± 21.2	-
starved 3d	24.0 ± 8.7	-	65.4 ± 3.6	-
starved 3d, refed 3h	11.3 ± 0.9	17.4 ± 2.2	41.4 ± 1.8	49.4 ± 7.9
starved 3d, refed 6h	19.1 ± 1.4	12.7 ± 2.0	53.2 ± 2.5	44.5 ± 12.6
starved 3d, refed 48h	217.0 ± 40.4	353.9 ± 171.5	132.5 ± 39.8	165.2 ± 7.8

[a]This is a continuation of the experiment described in the legend to Table II. Dehydrogenase activities are mU/mg protein.

[b]Dosage of 2.5 mg/100 g body weight.

β-galactosidase activity (Table II). Percent free hexosaminidase activity was unaltered by this treatment. Liver percent free β-galactosidase activity of rats injected with corticosterone was not altered during starvation-refeeding. The ability of rats to induce G6DPH and 6PGDH in response to starvation and refeeding for 2 days was unaltered by injection of corticosterone during the refeeding period. This result is shown in Table III. Liver and body weights for this experiment are shown in Table IV.

The effect of glucose injection into fed rats on liver percentage free lysosomal enzyme activity was examined to determine whether glucose was the mediator of the fragility increases observed during refeeding. These results are shown in Table V. Three hours after a single injection of glucose at (750 mg/100 g body weight), there was a significant 3.5, 2.7, and 1.5-fold increase in the percentage free activity of hexosaminidase, β-galactosidase, and β-glucuronidase respectively above water injected control activities at 3 hours after injection. Water injection did not affect the percentage of free lysosomal enzyme activity. Fragility returned to normal one day after glucose injection and remained at basal levels for the remaining two days of the experiment. A single injection of glucose into fed rats had no significant effect on the liver activities of G6PDH and 6PGDH (Table V). Liver and body weights for this experiment are alsoshown in Table V.

Nuclear lysosomal enzyme activity during starvation-refeeding. Some characteristics of the nuclei isolated by the method described are shown in Table VI. The protein/DNA ratio of purified nuclei of starved-refed rats showed a slight but significant increase (6.8%) in the first 1.5 hours after refeeding (Table VI). Control fed rats had higher nuclear

Table IV. Liver and Body Weights of Normal and Corticosterone Injected Rats During Starvation-Refeeding.[a]

	Final Body weight (g)		Liver Weight / Body Weight (%)	
	corticosterone injected[b]		corticosterone injected[b]	
Treatment	-	+	-	+
fed rat chow	243 ± 28	-	4.23 ± 0.45	-
fed glucose diet	193 ± 1	-	4.18 ± 0.01	-
starved 3d	203 ± 4	-	3.59 ± 0.17	-
starved 3d, refed 3h	185 ± 13	162 ± 32	3.51 ± 0.45	3.50 ± 0.24
starved 3d, refed 6h	183 ± 4	171 ± 19	3.83 ± 0.20	4.03 ± 0.19
starved 3d, refed 48h	212 ± 14	213 ± 4	4.84 ± 0.49	4.93 ± 0.09

[a]This is a continuation of the experiment described in the legend to Table II. Values are means ± S.D. of 3 rats.

[b]Dosage of 2.5 mg/100 g body weight.

Table V. The Effect of Glucose Injection on Liver Lysosome Fragility and Induction of Dehydrogenases in Fed Rats.[a]

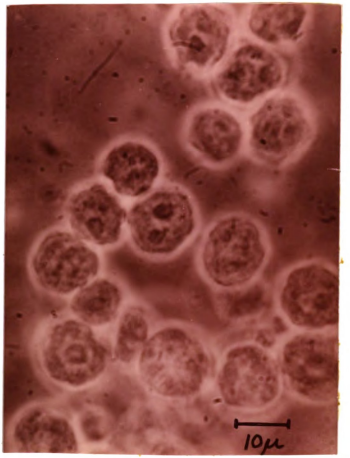
Property	Glucose[a]	Time After Injection: 3 h	1 day	2 days	3 days
Final Body Weight (g)					
	-	355 ± 18	-	319 ± 13	320 ± 7
	+	306 ± 46	311 ± 12	326 ± 17	345 ± 3
Liver Weight/Body Weight (%)					
	-	3.99 ± 0.05	-	4.06 ± 0.15	3.98 ± 0.05
	+	4.34 ± 0.13	3.72 ± 0.25	3.92 ± 0.18	4.28 ± 0.30
Liver Enzyme Activities					
Hexosaminidase					
	-	2.90 ± 0.46[a]	-	3.07 ± 0.52[a]	2.55 ± 0.19[a]
	+	10.1 ± 2.82[b]	2.86 ± 0.29[a]	3.31 ± 0.22[a]	3.40 ± 0.52[a]
β-Galactosidase					
	-	8.64 ± 0.49[a]	-	8.90 ± 0.55[a]	8.29 ± 0.85[a]
	+	23.5 ± 6.0[b]	9.27 ± 0.62[a]	9.44 ± 0.39[a]	9.63 ± 0.78[a]
β-Glucuronidase					
	-	19.1 ± 3.0[a]	-	17.3 ± 3.4[a]	15.4 ± 0.8[a]
	+	28.9 ± 5.7[b]	15.5 ± 2.3[a]	15.1 ± 1.3[a]	17.5 ± 4.0[a]
G6PDH					
	-	23.5 ± 1.7[a]	-	20.4 ± 2.8[a]	19.6 ± 5.5[a]
	+	25.6 ± 9.9[a]	22.6 ± 3.9[a]	19.0 ± 4.7[a]	18.7 ± 2.4[a]
6PGDH					
	-	80.0 ± 4.8[a]	-	78.9 ± 5.0[a]	71.3 ± 4.6[a]
	+	78.5 ± 5.8[a]	78.4 ± 4.5[a]	78.4 ± 7.8[a]	71.3 ± 4.9[a]

[a]Male Holtzman rats weighing initially 300-340 g were fed a rat chow diet throughout. Rats were injected i.p. with distilled water (-) or a 50% glucose solution (+) at 1.5 ml/100 g body weight at 0 hour and sacrificed at the time indicated. Livers were pepared and analyzed as described in Materials and Methods. Hexosaminidase, β-galactosidase, and β-glucuronidase values are percent free which is the activity/ml of the post-lysosomal (22,000 x g - 15 min) supernatant as a percent of the activity/ml of the post-nuclear (800 x g - 15 min) supernatant. G6PDH and 6PGDH activities are mU/mg protein. Values are means ± S.D. of 4 rats.

Table VI. Characteristics of Purified Rat Liver Nuclei.[a]

Treatment	Protein/DNA ratio		
	WH	PN	WH/PN
fed rat chow	49.3 ± 2.5[b]	2.75 ± 0.07[c]	17.9
fed glucose diet	48.6 ± 1.2[b]	2.65 ± 0.07[b,c]	18.3
starved 3d	37.1 ± 1.4[a]	2.36 ± 0.11[a]	15.7
starved 3d, refed 1.5h	36.0 ± 1.6[a]	2.52 ± 0.08[b]	14.3
starved 3d, refed 3.5h	35.6 ± 2.7[a]	2.52 ± 0.04[b]	14.1

[a]This is a continuation of the experiment described in the legend to Figure 3. Whole liver homogenates (WH) were unfractionated and the purified liver nuclear fraction (PN) was prepared as described in Materials and Methods. DNA concentrations ranged from 0.56 to 0.85 mg/ml for WH and 1.9 to 4.0 mg/ml for PN. Values are means ± S.D. of 4 rats except "fed glucose diet" which was from 3 rats.



protein/DNA ratios than nuclei of starved rats. This was a reflection of the higher protein content of livers from well fed rats (Table VI) compared with the protein content of starved rat liver. Nuclei were 8-10 μm in diameter with nucleoli plainly visible.

Nuclear lysosomal enzyme activities of rat liver during starvation-refeeding are shown in Figure 3. Liver nuclei from eleven day glucose diet fed rats showed no differences in specific activities of cathepsin D, hexosaminidase, and acid phosphatase compared with the same lysosomal enzyme activities of nuclei from control rats fed a chow diet. Starvation for 3 days resulted in a significant increase above control fed levels for liver nuclear hexosaminidase specific activity but not for cathepsin D or acid phosphatase specific activity. Refeeding starved rats a high carbohydrate diet lowered liver nuclear acid phosphatase activity to levels significantly below those of chow diet fed rats but not below those of 0 hour refed rats. Nuclear cathepsin D and hexosaminidase activities were not significantly altered from starvation levels by refeeding the high carbohydrate diet. Liver and body weights during this experiment are shown in Table VII.

Nuclear lysosomal activity was measured after injection of insulin into fed streptozotocin diabetic rats (Figure 4). Acid phosphatase activity decreased significantly 10 minutes after insulin injection then returned to normal by 60 minutes after injection. Nuclear cathepsin D activity did not vary significantly throughout the injection period. Diabetic rats which were starved and refed the high carbohydrate diet, with or without insulin supplementation at doses sufficient to induce G6PDH, 6PGDH, and ME (see Chapter 4 of this dissertation), showed no significant variation in nuclear acid phosphatase or percent free acid

Figure 3. Lysosomal Enzyme Activity of Liver Nuclei from Starved-Refed Rats. This is a continuation of the experiment shown in Table VI. Male Holtzman rats weighing initially 150-170 g were used. Controls were fed rat chow (C) or the high glucose diet (G) (see Materials and Methods) for 12 and 11 days respectively. "Refed" animals were starved for 3 days, refed the high glucose diet at 0 hour, and sacrificed at the times indicated. Purified liver nuclei were prepared and analyzed for lysosomal hydrolase activities, DNA, and protein content as described in Materials and Methods. Units: cathepsin D (method 1), μg tyrosine equivalents/hr/mg DNA; hexosaminidase, nmoles p-nitrophenol/hr/mg DNA; acid phosphatase (method 1) nmoles Pi/hr/mg DNA. Values are mean ± S.D. for 4 rats except G which was from 3 rats.

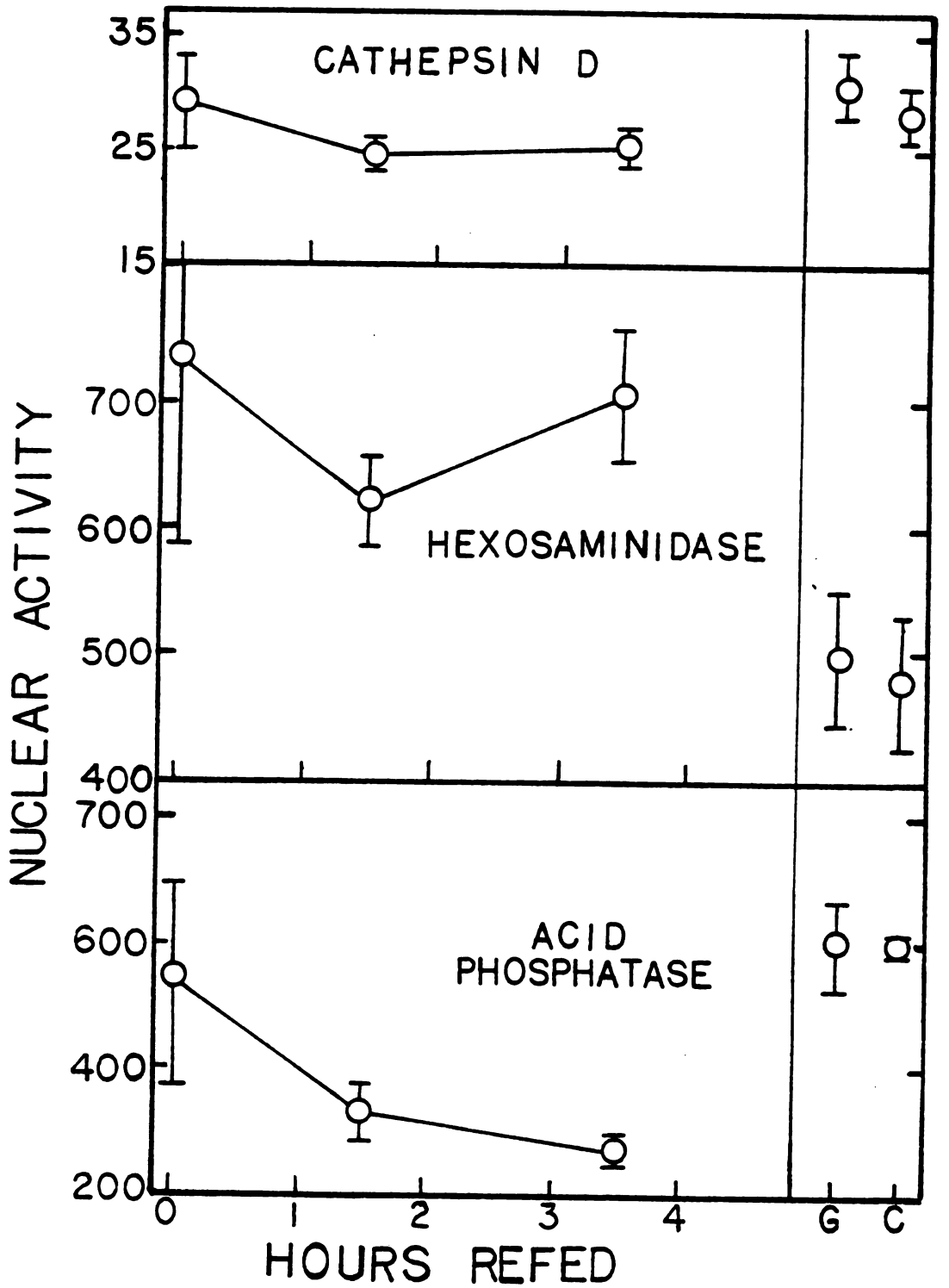


Figure 3. Lysosomal Enzyme Activity of Liver Nuclei from Starved-Refed Rats.

Table VII. Liver and Body Weights of Starved-Refed Rats.[a]

Treatment	Final Body weight (g)	$\frac{\text{Liver Weight}}{\text{Body Weight}}$ (%)
fed rat chow	271 ± 20	3.82 ± 0.30
fed glucose diet	258 ± 6	3.75 ± 0.19
starved 3d	180 ± 19	2.88 ± 0.05
starved 3d, refed 1.5h	204 ± 14	3.09 ± 0.14
starved 3d, refed 3.5h	202 ± 15	3.14 ± 0.12

[a]This is a continuation of the experiment described in the legend to Figure 3. Values are means ± S.D. of 4 rats except "fed glucose diet" which was from 3 rats.

Figure 4. Effect of Insulin Injection on Lysosomal Enzyme Activity of Liver Nuclei from Fed Streptozotocin Diabetic Rats. Male Holtzman rats weighing 130-200 g were made diabetic by i.v. injection of 65 mg streptozotocin/kg body weight. Four days later, at 0 hour, rats were injected i.p. with insulin at 3 U/100 g body weight (●) or saline (○) and sacrificed at the times indicated. Purified liver nuclei were prepared and analyzed for acid phosphatase (method 2) or cathepsin D (method 2) activities as described in Materials and Methods. Acid phosphatase and cathespin D activities were determined in two separate experiments of the same design. Activity units: acid phosphatase, nmoles Pi/hr/mg DNA; cathepsin D, cpm/90 min/mg DNA. "C" is the purified nuclear activity in livers from normal, non-diabetic, uninjected rats. All rats were fed rat chow throughout the experiment. Values are means ± S.D. for 4 animals.

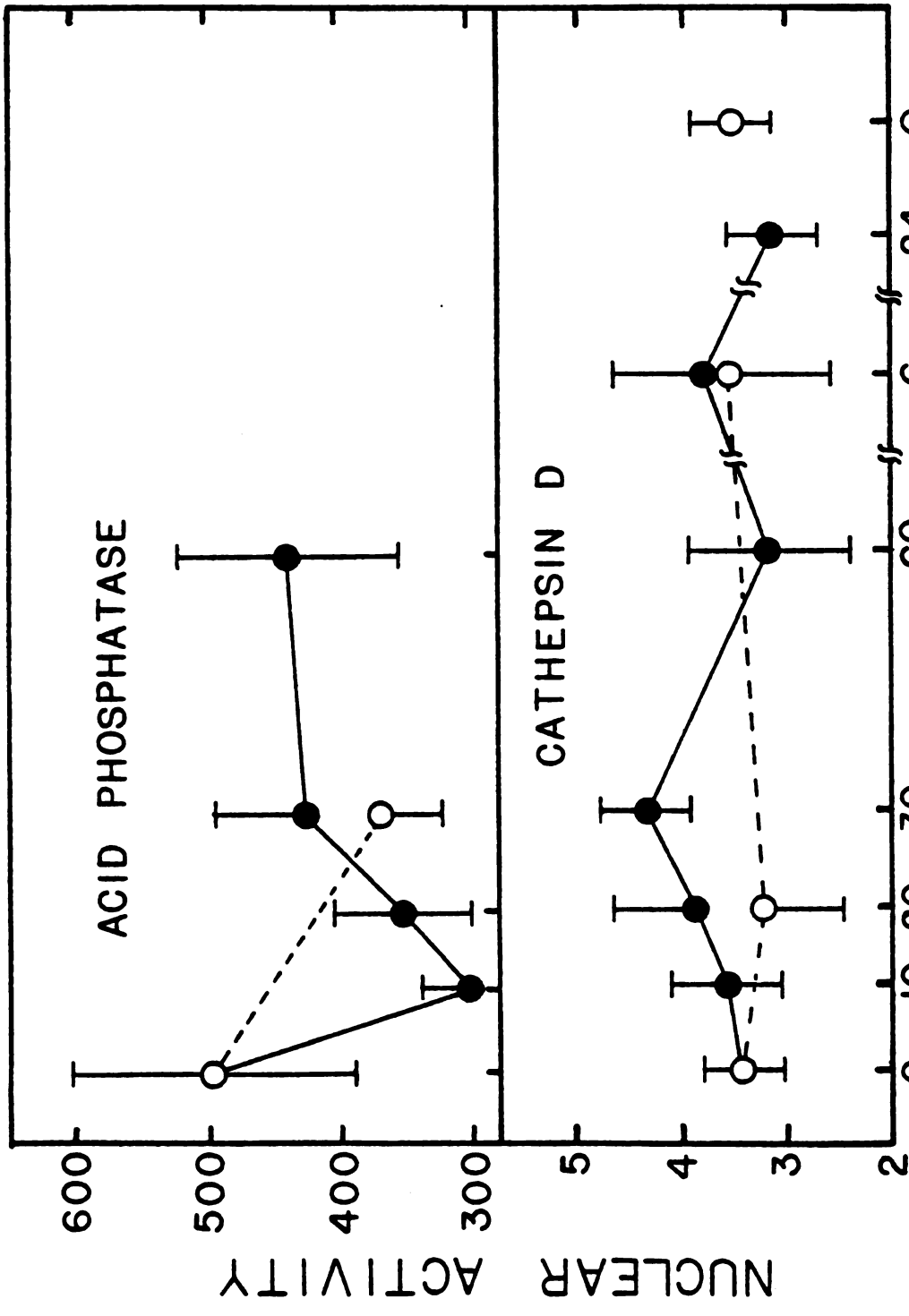


Figure 4. Effect of Insulin Injection on Lysosomal Enzyme Activity of Liver Nuclei from Fed Streptozotocin Diabetic Rats.

phosphatase activity (Table VIII). Non-diabetic control (normal) rats starved for three days then refed the high carbohydrate diet for 1 hour showed no significant alteration in liver nuclear acid phosphatase activity compared with liver nuclear acid phosphatase activity of 0 hour refed rats (Table VIII, Experiments 1 and 2).

Isolated hepatocytes were used to examine the effects of glucose, insulin, glucose + insulin, and serum from starved/1 hour refed rats on nuclear acid phosphatase activity. Characterization of this system is shown in Figure 5. As shown in this figure, viability and cellular integrity (determined by absence of leakage of cytosolic lactate dehydrogenase into the medium) were maintained throughout incubations. Glucose (4 mg/ml), insulin (40 mU/ml), and serum (17% final concentration) from control or 3 day starved 1 hour refed rats were without effect on nuclear acid phosphatase activity (data not shown).

Table VIII. Effect of Starvation-Refeeding and Insulin Injection on Liver Total, Percent Free, and Nuclear Acid Phosphatase Activity in Normal and Streptozotocin Diabetic Rats. Male Holtzman rats weighing 200-250 g were made diabetic by i.v. injection of streptozotocin (65 mg/kg body weight). Beginning one week after injection, rats were starved for three days then refed the high glucose diet (0 hour) for the times indicated with or without insulin supplementation as indicated. In experiment 1, a single i.p. injection of regular insulin was given at 3U/100 g body weight 30 min after initiation of refeeding. In experiment 2, insulin zinc suspension (Lente insulin) was injected s.c. at 15 U/100 g body weight at the time of refeeding. At the times indicated, animals were sacrificed and livers were prepared and analyzed for acid phosphatase (method 2) as described in Materials and Methods. Values are means ± S.D. of 8 and 4 rats in experiments 1 and 2 respectively. Acid phosphatase activity (method 2) for whole liver homogenates (WH) is μmoles Pi/hr/mg DNA and for purified liver nuclei (PN) is nmoles Pi/hr/mg DNA. Percent free is the activity/ml of the post-lysosomal (22,000 x g - 15 min) supernatant as a percent of the activity/ml of the post-nuclear (800 x g - 15 min) supernatant.

Table VIII. Effect of Starvation-Refeeding and Insulin Injection on Liver Total, Percent Free, and Nuclear Acid Phosphatase Activity in Normal and Streptozotocin Diabetic Rats.

Expt.	Treatment	Acid Phosphatase Activity PN	WH
1.	normal, starved 3d	343.9 ± 69.0[a]	99.7 ± 10.6[a]
	normal, starved 3d, refed 1h	381.3 ± 104.5[a]	91.0 ± 8.7[a]
	diabetic, starved 3d	371.0 ± 80.6[a]	79.9 ± 19.7[a]
	diabetic, starved 3d, refed 1h	351.5 ± 43.2[a]	80.7 ± 17.1[a]
	diabetic, starved 3d, + insulin	315.9 ± 48.6[a]	81.9 ± 11.3[a]
	diabetic, starved 3d, refed 1h + insulin	302.4 ± 58.5[a]	87.3 ± 8.2[a]
		PN	% Free
2.	normal, starved 3d	392.9 ± 81.8[b]	---
	normal, starved 3d, refed 1h	307.0 ± 47.8[b]	---
	diabetic, starved 3d, + insulin	207.5 ± 28.1[a]	22.1 ± 2.2[a]
	diabetic, starved 3d, refed 0.5h + insulin	229.8 ± 46.9[a]	20.0 ± 1.4[a]
	diabetic, starved 3d, refed 1h + insulin	193.4 ± 43.5[a]	21.2 ± 1.9[a]
	diabetic, starved 3d, refed 2h + insulin	211.6 ± 40.4[a]	16.4 ± 0.7[a]
	diabetic, starved 3d, refed 3h + insulin	219.5 ± 36.5[a]	18.4 ± 3.8[a]
	diabetic, starved 3d, refed 5h + insulin	263.5 ± 47.6[a]	17.8 ± 3.5[a]

Figure 5. Characterization of Suspension Cultures of Hepatocytes Isolated from Starved Rats. Male Holtzman rats weighing 300-330 g were starved for three days then used for hepatocyte preparation as described in the Materials and Methods section of Chapter 4 of this dissertation. Cells were suspended to a density of 3 x 10^6/ml in serum-free MEM with non-essential amino acids. Four ml of cell suspension was placed in 25 ml plastic Erlenmeyer flasks, gassed 30 sec with water saturated 95% O_2/5% CO_2, stoppered, and incubated at 37°C with shaking at 100 oscillations/min. At the times indicated cells were removed from medium by centrifugation and samples analyzed as described in Materials and Methods. Viability was determined as the percentage of cells excluding 0.2% trypan blue in four minutes. Acid phosphatase activities are μmoles Pi/hr/mg DNA for whole homogenates (WH) and nmoles Pi/hr/mg DNA for whole purified nuclei (PN). Lactate dehydrogenase (LDH) activity is μmoles NADH utilized/min/ml medium. Values are single determinations or means ± S.D. for 3 flasks of cells.

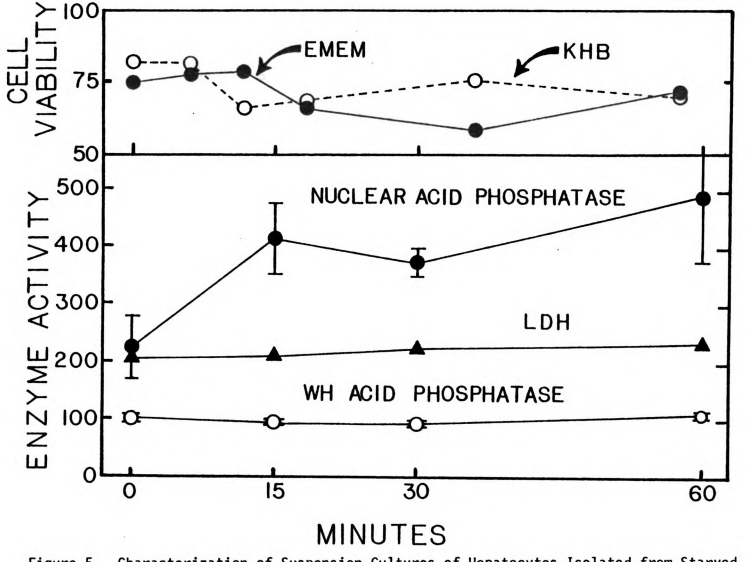


Figure 5. Characterization of Suspension Cultures of Hepatocytes Isolated from Starved Rats.

DISCUSSION

The previous finding (7) that starvation/refeeding is accompanied by a transient fragility of liver lysosomes was reconfirmed in this work (Figure 1A and 1B and Table II, control values). Although the times of peak lysosome fragility are not in exact agreement between these two reports, that is probably due to the choice of time points; the present study included a 9 hour refed point whereas the previous study (7) did not. Determination of the exact peak time would require a further examination.

Stabilization of lysosomes with corticosterone (Table II) was without effect on the liver's capacity to induce G6PDH and 6PGDH (Table III) in response to a starvation/refeeding stimulus. Labilization of lysosomes by glucose (Table V) or hydrocortisone injection (Figure 1) during the starvation/refeeding regimen was in itself ineffective in promoting an induction of G6PDH and 6PGDH. The labilization of lysosomes by hydrocortisone injection despite the reported stabilizing effect of hydrocortisone on lysosomes (18), could be explained by the fact that the vehicle used was saline with Tween 80 (1 drop Tween 80/5 ml saline) according to Zarrow et al. (19). These results suggest that the observed transient increase in lysosomal fragility (ref. 7, and Figure 1), while associated with the induction of G6PDH and 6PGDH during starvation/refeeding, is not an integral part of the induction signal. The absence of a transient fragility of mitochondria during the starvation/refeeding regimen would indicate the specificity of the lysosome response. The surprisingly large percent free fumarase activity (50%) is in agreement with the findings of Nakashima et al. (20). These authors also saw a decline in

cytosolic fumarase after feeding rats a 60% glucose diet.

The increased susceptibility of lysosomes to breakage and release of lysosomal hydrolases during starvation/refeeding would indicate an altered state of the lysosomal membranes of refed rats. This observation is in agreement with recent observations that insulin, which is elevated in the circulation during refeeding, is internalized and associated with the Golgi apparatus and lysosomes (21,22). In this regard, Verity has suggested that fusion of lysosomal membranes with other membranes is manifested by a transient labilization at the membrane interface (23). Fusion of an endocytic vacuole of insulin receptor molecules with lysosomes during starvation/refeeding might therefore account in part for the observed (7) transient fragility of lysosomes during refeeding. Other factors such as dietary glucose (see Table V) may also account in part for the fragility seen during refeeding.

In the present investigation, nuclear lysosomal enzyme activities were not altered in a consistent fashion by starvation/refeeding (Figure 3 and Table VIII) or insulin injection of diabetic starved or fed rats (Figure 4, Table VIII). This is in disagreement with the studies of Mak and Wells (8). Several attempts to obtain an increase in nuclear acid phosphatase activity at one hour after refeeding a high carbohydrate diet to 3 day starved rats have failed. Results of two such attempts are shown in Table VIII. It should be noted that the nuclear acid phosphatase activities of Mak and Wells (8) are on the order of 500-700 nmoles Pi/hr/mg DNA whereas the activities listed here for normal rats, run under the same assay conditions (method 2) in the later studies, are on the order of 300-500 nmoles Pi/hr/mg DNA. The overall purity of the

two preparations, as judged by the nuclear protein/DNA ratio, are in close agreement. The discrepancies between these reports would indicate that an alternate approach is required to determine the possible association of lysosomes with nuclei during starvation/refeeding. One would be a detailed electron microscopic examination of livers of starved-refed rats.

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Chapter IV

Induction of Lipogenic Enzymes in Rat Liver and in Primary Cultures of Adult Rat Hepatocytes.

SUMMARY

The contributions of insulin and glucose to the induction of hepatic glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and malic enzyme (ME) were examined *in vivo* and *in vitro*. Normal and streptozotocin diabetic male rats weighing 200-250g were starved for three days, then refed a high carbohydrate diet (68.9% glucose, 20% casein, 5% corn oil, 5% salt mix, 1% vitamin mix, and 0.1% choline chloride) for four days.

In diabetic rats, the induction responses of G6PDH, 6PGDH, and ME proceeding from the three day starved to the four day refed state, were 10, 22, and 13% respectively of those observed in similarly treated normal rats. This difference for diabetic rats was not the result of inadequate glucose consumption since starved-refed diabetic rats consumed 59% more diet than starved-refed normal rats. Serum immunodetectable insulin concentrations of diabetic rats averaged 41% lower than those of normal rats and increased 2.6 fold in response to refeeding in both normal and diabetic rats. The latter insulin response was ineffective in lowering serum glucose concentrations to normal levels. Supplementation of starved diabetic rats with insulin during the four days of refeeding controlled serum glucose levels and restored the induction of G6PDH, 6PGDH, and ME to levels above normal.

Hepatocytes were isolated from 3-day starved male rats weighing 200-250 g and incubated in serum-free Dulbecco's medium containing twice normal concentration of amino acids (DMEA) in cultures of 3 x 10^6 cells per dish. G6PDH specific activity increased 2 to 3-fold in 48 hour control incubations in DMEA and increased an additional 3.5-fold in the presence of 42 mU/ml insulin, 1 μM dexamethasone, and the absence of medium glucose. The effects of insulin and dexamethasone on the induction of G6PDH were dose dependent and additive; each contributing about one half of the total response. The increase in G6PDH specific activity by insulin and dexamethasone were independent of DNA synthesis. 6PGDH and ME specific activities decreased during the 48 hour control incubation in DMEA. Insulin, but not dexamethasone, prevented this decrease in activity and increased 6PGDH activity 20% above 0 hour levels. Cells incubated in DMEA with glucose up to 27.3 mM in the absence of hormones showed no increase in G6PDH, 6PGDH, and ME activities. Addition of triiodothyronine (15 μM) to cells incubated for 48 hour blunted the insulin and dexamethasone stimulated increases in G6PDH and 6PGDH activities and slightly (8%) increased ME specific activity. The results presented here indicate that glucose alone is not sufficient to induce these liver lipogenic enzymes but that insulin is required for the induction of G6PDH, 6PGDH and ME *in vivo* and for G6PDH and 6PGDH *in vitro*.

INTRODUCTION

The activities of the liver lipogenic enzymes G6PDH[1], 6PGDH, and ME increase above normal levels (overshoot) during the first few days of feeding a high carbohydrate diet to rats previously starved (1). A number of nutritional and hormonal agents such as dietary carbohydrate (2-8), lipid (4,9-12), and protein (5,8,13,14) as well as insulin (15-21), glucocorticoids (22-25), thyroid hormones (19,20,26), and sex steroids have been examined as regulators of the induction[2] of these enzymes.

At present there is disagreement about the roles of glucose and insulin in the induction of G6PDH. There is evidence that insulin is required for the induction of this enzyme in vivo (16,17,21), and in isolated hepatocytes in the presence of fetal bovine serum, dexamethasone, and glucose (22). However, other reports indicate that dietary carbohydrate is the primary inducing stimulus and that insulin serves to indirectly promote induction by glucose through an increase in appetite (2,4) or an increase in transport of glucose into liver cells (6). Glucocorticoids are required for the overshoot induction of G6PDH and 6PGDH in vivo (23-26). Recently, it was demonstrated that the mechanism by which the combination of a number of metabolite and hormone additions

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[1]Abbreviations: DME = Dulbecco's modified Eagle's medium; DMEA = DME supplemented with amino acids as described in Materials and Methods; FBS = fetal bovine serum; MEM = modified Eagle's medium; G6PDH = D-glucose 6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49; 6PGDH = 6-phospho-D-gluconate:$NADP^+$ 1-oxidoreductase (decarboxylating), EC 1.1.1.44; ME = L-malate:$NADP^+$ oxidoreductase, EC 1.1.1.40; T_3 = triiodothyronine.

[2]Induction is here defined as an increase in enzyme specific activity.

to isolated hepatocytes in culture (27,28) or starvation-refeeding *in vivo* (29,30) caused an increase in G6PDH activity was through messenger RNA transcription and post-transcriptional control, whereas induction of 6PGDH (31,32) and ME (33) were stoichiometrically related to increases in transcription.

While these investigations have provided much information about control of lipogenesis enzyme induction, there is a need to distinguish between the individual contributions of glucose, insulin, glucocorticoids, and other hormones, as signals for the induction of these enzymes.

The purpose of the present investigation was twofold: (1) to re-examine the requirement for insulin *in vivo* as an inducer of the lipogenic enzymes, G6PDH, 6PGDH, and ME during the transition from starvation to refeeding and to determine if this requirement is an indirect effect on diet consumption and (2) to examine the direct effects of glucose and insulin as well as dexamethasone and triiodothyronine, singly and in combination, on the isolated hepatocytes as a requirement for the induction of G6PDH, 6PGDH, and ME.

MATERIALS AND METHODS

Materials. Collagenase (EC 3.4.99.5) CLS II and Statzyme glucose determination kits were obtained from Worthington Biochemical Co., Freehold, N.J. Insulin, Regular and Lente Iletin, 100 units/ml was from Eli Lilly and Co., Indianapolis, IN. DME with 4.0 mM L-glutamine, 1.0 mM sodium pyruvate and 5.6 mM glucose in powdered form; MEM non-essential amino acids solution (100X), MEM amino acids solution with 100 mM L-glutamine (50X), penicillin-streptomycin solution (10,000 U/ml penicillin - 10,000 µg/ml streptomycin), and fetal bovine serum were obtained from Grand Island Biological Co., Grand Island, NY. Trypan blue was from Matheson, Coleman and Bell Co., Norwood, OH. Streptozotocin was a gift from Dr. W.E. Dulin of the Upjohn Co., Kalamazoo, MI. Aphidicolin was a gift from Dr. John D. Douros, Developmental Therapeutics Program, Chemotherapy, National Cancer Institute to Dr. John A. Boezi of this department. All other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Animals. Male rats weighing 200-250 g were purchased from the Holtzman Co., Madison, WI, and housed as previously described (34). Diabetes was produced by injection of streptozotocin (65 mg/kg body weight) dissolved in 0.1 M sodium citrate buffer, pH 4.5 at a concentration of 65 mg/ml into the femoral vein of ether anesthetized rats within 10 min of solution preparation. One week after streptozotocin injection, blood was collected into heparinized capillary tubes and centrifuged to separate plasma from cells. All animals judged to be diabetic had plasma glucose levels $\geq$ 22 mM. Normal or diabetic rats were starved for three days with access to water and refed the high glucose diet: 68.9%

glucose, 20% casein, 5% corn oil, 5% salt mix, 1% vitamin mix, and 0.1% choline chloride prepared as described previously (35). At various times after refeeding, animals were killed by decapitation and trunk blood was collected in unheparinized centrifuge tubes. Serum was collected by centrifugation and stored at -80°C until glucose and insulin analyses were performed. Livers were removed and placed in 0.25 M sucrose, 2 mM $MgCl_2$ at 0-4°C. All subsequent operations were performed at 0-4°C. Four grams of liver were minced, mixed with 23 ml of 0.25 sucrose, 2 mM $MgCl_2$ and homogenized and centrifuged at 105,000 x g for 1 hr as described previously (31) to obtain a supernatant fraction for G6PDH, 6PGDH, and ME analysis.

Media preparation. Calcium- and magnesium-free Krebs-Henseleit bicarbonate buffer, hereafter designated KHB(-Ca^{2+} and Mg^{2+}), used in each perfusion contained a lower concentration of $NaHCO_3$ than the original formulation and was prepared by combining the following: 600 ml 0.9% NaCl, 24 ml 1.15% KCl, 6 ml 2.11% KH_2PO_4, 102 ml 1.3% $NaHCO_3$ and 40 ml H_2O. This solution was sterilized in an autoclave. Before use the pH was brought to 7.4 by gassing with 95% O_2/5% CO_2 for 1 hour at 10% maximum flow (Model 10A3135 flow meter, Fischer and Porter Co., Warminster, PA) while the temperature was equilibrated to 37°C. KHB(-Ca^{2+} and Mg^{2+}) was then supplemented to 100 U/ml penicillin-100 µg/ml streptomycin. DME was prepared from powder, supplemented with 100 U/ml penicillin-100 µg/ml streptomycin and sterilized by membrane filtration. Before use DME was supplemented to twice normal concentration with MEM amino acids and MEM non-essential amino acids and titrated to pH 7.6 by addition of 1.3 ml 1 N NaOH per 100 ml DME. This solution is hereafter referred to as DMEA. DMEA contains a basal level

of 5.6 mM glucose. In one experiment (Figure 2) a glucose-free modified DME was prepared as follows (all components are as indicated per liter of solution): 6.4 g NaCl, 0.4 g KCl, 0.2 g $CaCl_2$, 97.6 mg $MgSO_4$, 125 mg $NaH_2PO_4 \cdot H_2O$, 40 µg $FeCl_3$, 15 mg phenol red, 110 mg sodium pyruvate, 30 mg glycine, 42 mg serine, 40 ml MEM amino acids, and 40 ml MEM vitamin mix. This medium was then supplemented with MEM amino acids, pencillin and streptomycin, and titrated to pH 7.6 as done for DMEA. This medium did not contain glucose as determined using the statzyme glucose kit. This method is based on the use of hexokinase, G6PDH and the reduction of $NADP^+$ at 340 nm and 25°C in a Gilford 3500 spectrophotometer.

Hepatocyte isolation. Hepatocytes were isolated by a modification of the method of Seglen (36). Following ether anesthesia and abdominal incision as described (36), two loose ligatures were placed around the portal vein and one around the inferior vena cava just proximal to the right renal vein. Each rat was then heparinized by injection into the vena cava of 0.1 ml/100 g body weight of a 5,000 IU/ml heparin solution prepared in 0.9% saline. The portal vein was cannulated with PE-160 Intramedic polyethylene tubing (Clay Adams, Parsippany, N.J.) and secured 1 cm from the liver so the tip of the tubing was at the edge of the liver. When perfusion was begun, the inferior vena cava was ligated and the superior vena cava was severed 2 cm above diaphram. Single-pass liver perfusion was begun at 35 ml/min with an inital 600 ml KHB ($-Ca^{2+}$ and Mg^{2+}), continuously gassed with 95% O_2/5% CO_2 as described by Seglen (36), and heated to 37°C prior to entry into the liver by passage through the coils of a thermostated reflux type condensor (Bantam-ware, Kontes Glass Co., Vineland, N.J.). During perfusion

the liver was carefully dissected from the animal and placed on a 7 cm diameter, 242 μm mesh nylon disc supported by a 2 mm mesh circular stainless steel wire platform in a 37°C water-jacketed glass dish. The liver was perfused single-pass until the remaining perfusate volume was 130 ml. At this time 3 ml of 1.22% $CaCl_2$ solution containing 65 mg collagenase was mixed with the perfusate and cyclic perfusion was continued for 25-30 min. The dispersed liver cells were further gently disrupted with a stainless steel spatula in a 50 ml Nalgene beaker, then incubated in 25-50 ml of collagenase-perfusate for 5 min at 37°C in an atmosphere of 95% O_2/5% CO_2 with shaking at 100 oscillations/min. Only plastic containers and pipettes were used when handling hepatocytes. After incubation, hepatocytes were separated from undispersed liver by filtration through a 242 μm mesh nylon cone and brought to a volume of 40-45 ml with KHB(-Ca^{2+} and Mg^{2+}). Parenchymal cells were pelletted and separated from Kupffer cells by centrifugation at 50 x g for 2 min at room temperature. The parenchymal cell pellet was gently resuspended with a rubber policeman and washed once with KHB(-Ca^{2+} and Mg^{2+}) and once with DMEA. Cell aggregates which settled out in 30s at unit gravity were removed with a pasteur pipette prior to centrifugation. At the DMEA wash, cell number and viability were assessed using a Neubauer hemacytometer by determing the percentage cells excluding dye in a 0.2% trypan blue solution at pH 7.4. Cells were finally resuspended in DMEA to a density of 2 x 10^6 viable cells/ml.

Hepatocyte culture. Tissue culture dishes of 60 mm diameter (Falcon Plastics, Oxnard, CA or Corning, Corning, N.Y.) coated with FBS as described by Seglen and Fossa (37) were prepared the day before an experiment and stored at 4°C. DMEA was used throughout because this medium and

high levels of amino acids are known to support protein synthesis in cultures of isolated hepatocytes (38-41). Three to four hours prior to cell plating, 1.5 ml DMEA containing various hormone and metabolite additions was added to the FBS-coated dishes and equilibrated to 37°C in an air atmosphere supplemented to 5% CO_2. Experiments were initiated by addition of 1.5 ml of cell suspension to dishes. At 24 hour intervals, unattached or weakly attached cells were removed by mild pipetting of the incubation medium against the bottom of the dish three or four times using a Pasteur pipette. The old medium and dead cells were then replaced by fresh medium.

Cell harvest and fractionation. Cells were harvested from the tissue culture dishes as follows. Unattached and weakly attached cells were removed from the dishes as described. Remaining attached cells were rinsed once with 1.5 ml of 0.9% NaCl solution and then again with repetitive mild pipetting against the bottom of the dish until few cells were released. After removal of the second rinse, 1.5 ml of 0.25 M sucrose, 2 mM $MgCl_2$ at 4°C was added to the dish. Cells were detached from the dish using a rubber policeman and decanted into 1.4 cm (i.d.) x 10.3 cm polycarbonate tubes. Dishes were rinsed with 0.5 ml 0.25 M sucrose, 2 mM $MgCl_2$ which was also added to the cells. Cells were then homogenized at 0-4°C with a Tekmar homogenizer as previously described (42). Homogenates were centrifuged for 30 min at 40,000 x g at 4°C and supernatants fractions collected for enzyme and protein analysis.

Analyses. G6PDH and 6PGDH activities were determined in the above supernatants by the spectrophotometric method previously described (42). Malic enzyme activity was determined by the method of Hsu and Lardy

(43). For G6PDH, 6PGDH, and ME, one unit (U) represents production of 1 μmole NADPH/min.

Protein analysis was performed by the fluorescamine method and DNA was determined by the ethidium bromide method as previously described (42).

Serum insulin concentration was determined using an insulin immunoassay kit (Amersham, Arlington Heights, IL) which closely resembles the double antibody method of Hales and Randle (44). The kit employes human insulin standards, guinea pig anti-insulin and rabbit anti-guinea pig serum protein.

Serum or plasma glucose levels were determined using the statzyme glucose kit.

All statistical methods were according to Steel and Torrie (45).

RESULTS

Induction of lipogenic enzymes in normal and diabetic rats. Table I shows the effects of diet consumption and insulin administration on the specific activities of three lipogenic enzymes, G6PDH, 6PGDH, and ME in the livers of 3 day-starved normal and streptozotocin diabetic rats. Induction of these enzymes by a 3 day starvation/4 day refeeding regimen is greatly impaired in diabetic rats. After three days of starvation, the liver specific activities of G6PDH, 6PGDH, and ME in diabetic rats did not differ significantly from the same activities determined by 3 day starved normal rats. Refeeding of a high carbohydrate diet to starved diabetic rats for four days resulted in significant elevations in liver specific activities of G6PDH, 6PGDH, and ME to values 2.3, 1.6, and 2.4 fold above activities found in the starved state. These elevations represented a return to normal levels rather than an overshoot, since mean activities of starved-refed diabetic rat liver did not differ significantly from activities present in normal rat liver. In contrast, the liver activities of these enzymes in starved refed normal rats were respectively 22.5, 7.4, and 18.1 fold elevated above activities determined in livers of starved normal rats. This represents a 14.8, 4.1, and 8.5 fold overshoot of normal levels. If the observed increases in liver G6PDH, 6PGDH, and ME specific activity for normal starved-refed rats are set at 100%, the diabetic rats were 10, 22, and 13%, respectively.

Administration of insulin (15 U Lente insulin/100 g body weight injected subcutaneous daily) to 3 day-starved diabetic rats during four days of refeeding was accompanied by a marked elevation in the activities of the three lipogenic enzymes examined. Liver activities of G6PDH, 6PGDH, and ME in insulin treated, starved-refed diabetic rats were 27.4,

Table I. Liver Lipogenic Enzyme Activities Of Normal and Streptozotocin-Diabetic Rats During Starvation-Refeeding. This description also applies to Table II and III. Male Holtzman rats weighing 200-250 g were made diabetic by intravenous injection of streptozotocin (65 mg/kg body weight). Beginning 1 week after injection, rats were starved for 3 days, then refed a high glucose diet (see Materials and Methods) for 4 days (experiment 1) or 1 hour (experiment 2) with or without insulin supplementation as indicated. In experiment 1 insulin zinc suspension (Lente insulin) was injected subcutaneous daily at 15 U/100 g body weight. In experiment 2, a single intraperitoneal injection of regular insulin was given at 3 U/100 g body weight 30 min after initiation of refeeding. Diet consumed is the average daily amount per 100 g final body weight in experiment 1 and total amount consumed in one hour in experiment 2. Animals were sacrificed by decapitation, trunk blood was collected in unheparinized centrifuge tubes, and livers were rapidly excised and placed in 0.25 M sucrose, 2 mM $MgCl_2$ on ice. Serum was collected after centrifugation. Liver homogenates, 15% (w/v) in 0.25 M sucrose, 2 mM $MgCl_2$, were centrifuged at 105,000 x g for 1 hour and supernatant fractions were analyzed for G6PDH, 6PGDH and ME activity at 30°C. Values are means ± S.D. of n rats per group. Means not having a common superscript are significantly different as determined by analysis of variance, least significant difference test, $p < 0.05$ and superscript notations of experiments 1 and 2 are independent.

Table I. Liver Lipogenic Enzyme Activities Of Normal And Streptozotocin-Diabetic Rats During Starvation-Refeeding.

Expt.	Treatment	n	Enzyme Activity (mU/mg protein) G6PDH	6PGDH	ME
1.	normal	4	24.9 ± 5.2[b]	60.6 ± 2.5[b]	22.6 ± 1.1[b]
	normal, starved 3d	5	16.4 ± 2.9[a]	33.7 ± 6.7[a]	10.6 ± 1.7[a]
	normal, starved 3d, refed 4d	5	369.8 ± 84.1[c]	248.6 ± 38.0[c]	191.4 ± 4.1[c]
	diabetic, starved 3d	5	11.8 ± 1.3[a]	32.0 ± 2.1[a]	7.4 ± 0.6[a]
	diabetic, starved 3d, refed 4d	4	27.3 ± 10.4[b]	52.0 ± 10.4[b]	18.0 ± 5.4[b]
	diabetic, starved 3d, refed 4d + insulin	5	747.5 ± 67.1[d]	363.3 ± 25.4[d]	316.5 ± 29.9[d]

7.0, and 17.6 fold elevated above activities determined in non-insulin treated, starved-refed diabetic rats and were 2.0, 1.5, and 1.7 fold elevated respectively above activities of starved-refed normal rats.

Diabetic rats were unable to properly dispose of incoming dietary glucose whereas normal rats had this capacity. Seven days after streptozotocin injection, serum glucose of rats rose from the normal level of 8.8 ± 0.4 (n = 4) to 30.8 ± 4.0 mM (n = 61). As indicated in Table II, three days of starvation of diabetic rats resulted in significantly reduced serum glucose levels. In normal rats, serum glucose levels dropped significantly from 8.8 to 6.1 mM after three days of starvation. Serum glucose levels of 3 day-starved normal and 3 day-starved diabetic rats then rose 2.0 and 3.3 fold, respectively, after refeeding the high carbohydrate diet for 1 hour. After 4 days of feeding the high carbohydrate diet, the serum concentration of glucose of normal rats returned to pre-starvation levels while the serum glucose concentration of diabetic rats remained elevated at 31.0 mM.

The mean concentrations of serum insulin in streptozotocin diabetic rats were in all cases lower than those of normal rats although these differences were not always statistically significant (Table II). In response to starvation, the serum insulin concentration of both normal and diabetic rats was lowered to 8 and 3 μU/ml, respectively, in experiment 1 and to 24 and 19 μU/ml, respectively, in experiment 2. Both normal and streptozotocin diabetic rats which had been starved for 3 days, then refed a high-carbohydrate diet for 1 hour, responded with a 2.6 fold elevation in serum insulin concentration above levels found prior to refeeding. Although the immunochemically detectable insulin response to refeeding in diabetic rats was proportional in amount to that

Table II. Serum Glucose and Insulin Concentrations of Normal and Streptozotocin-Diabetic Rats During Starvation/Refeeding. See legend to Table I.

Table II. Serum Glucose And Insulin Concentrations Of Normal And Streptozotocin-Diabetic Rats During Starvation-Refeeding.

Expt.	Treatment	n	Glucose (mM)	Insulin (μU/ml)
1.	normal	4	8.8 ± 0.4[b]	56 ± 23[d]
	normal, starved 3d	5	6.9 ± 0.8[a]	8 ± 2[b]
	normal, starved 3d, refed 4d	5	8.4 ± 0.7[b]	121 ± 77[e]
	diabetic, starved 3d	5	8.3 ± 1.7[a,b]	3 ± 2[a]
	diabetic, starved 3d, refed 4d	4	31.0 ± 14.0[d]	17 ± 5[c]
2.	normal, starved 3d	8	6.1 ± 0.8[b]	24 ± 14[a]
	normal, starved 3d, refed 1h	8	12.1 ± 1.6[c]	62 ± 16[b]
	diabetic, starved 3d	8	7.5 ± 1.2[b]	19 ± 9[a]
	diabetic, starved 3d, refed 1h	8	24.5 ± 7.1[d]	50 ± 12[b]
	diabetic, starved 3d, + insulin	8	2.9 ± 0.5[a]	>1600[c]
	diabetic, starved 3d, refed 1h + insulin	8	6.0 ± 2.3[b]	>1600[c]

of normal rats, the former was ineffective in controlling serum glucose (Table II). Insulin, when injected into streptozotocin diabetic rats during refeeding, was effective in lowering serum glucose. This indicates that peripheral tissues of diabetic rats were responsive to exogenous insulin and that the immunodetectable insulin of diabetic rats was either quantitatively inadequate or abnormal in hypoglycemic activity. After four days of feeding, normal and diabetic rat serum insulin levels were elevated above starvation levels (Table II, experiment 1), however refed normal rat serum insulin levels were 7-fold higher than those in refed diabetic rats.

During the four day refeeding period, diabetic rats consumed 60% more diet daily per 100 g final body weight than normal rats starved and refed in the same way (Table III). Daily insulin administration to starved-diabetic rats during the refeeding period lowered the average food consumed daily per 100 g body weight to a level which was still 23% above that of starved-refed intact rats (Table III). Liver weight per 100 g body weight was reduced by starvation and returned to normal values after refeeding in both normal and diabetic rats. In diabetic refed rats, supplementary insulin dramatically increased liver weights per 100 g body weight as compared with other groups. Livers of this group were fatty in appearance.

Despite an intake of dietary glucose which would normally promote an overshoot of lipogenic enzyme induction in the intact rat, starved-refed diabetic rats were incapable of overshoot induction unless supplemented with insulin. Insulin was not required for the return of enzyme activities from starvation to normal levels. However, since insulin was detectable immunochemically in diabetic rats and because it was uncertain

Table III. Diet Consumption And Liver Weight As Percent Body Weight Of Normal and Streptozotocin-Diabetic Rats During Starvation-Refeeding. See legend to Table I.

Table III. Diet Consumption And Liver Weight As Percent Body Weight Of Normal And Streptozotocin-Diabetic Rats During Starvation-Refeeding.

Expt.	Treatment	n	Diet Consumed (g)	Liver Weight (% body weight)
1.	normal	4	---	3.73 ± 0.11[b]
	normal, starved 3d	5	0	2.88 ± 0.16[a]
	normal, starved 3d, refed 4d	5	9.8 ± 1.8[a]	4.62 ± 0.26[c]
	diabetic, starved 3d	5	0	3.28 ± 0.13[a,b]
	diabetic, starved 3d, refed 4d	4	15.6 ± 3.4[c]	4.49 ± 0.39[c]
	diabetic, starved 3d, refed 4d + insulin	5	12.1 ± 0.8[b]	8.30 ± 0.64[d]
2.	normal, starved 3d	8	0	2.92 ± 0.13[a]
	normal, starved 3d, refed 1h	8	3.7 ± 1.6[a]	2.99 ± 0.15[a]
	diabetic, starved 3d	8	0	3.28 ± 0.19[b]
	diabetic, starved 3d, refed 1h	8	3.7 ± 2.7[a]	3.55 ± 0.26[b]
	diabetic, starved 3d, + insulin	8	0	3.29 ± 0.24[b]
	diabetic, starved 3d, refed 1h + insulin	8	2.7 ± 1.5[a]	3.51 ± 0.34[b]

whether insulin was acting directly on the liver cell alone or in concert with glucose or other signals, we selected the relatively less complicated isolated rat hepatocyte system in culture for further investigation of lipogenic enzyme induction.

Cell culture. Hepatocytes isolated by the methods described were routinely 90% viable as determined by exclusion of trypan blue with cell yields of 2 to 5 x 10^8 per rat. Hepatocytes maintained in culture as described attached to the FBS-coated dishes within the first 40 min after plating. After 24 hours of culture, 1/3 to 1/2 of the cells which were plated remained attached. After rinsing the cells gently as described, approximately 95% of the cells which remained on the plate excluded trypan blue and, of the cells removed from the tissue culture plate, 10% excluded trypan blue. There was very little cell death beyond the first day in culture. The presence of dexamethasone (1 μM) in the culture medium improved plating efficiency and increased cell aggregation into "trabecular aggregates" (36).

Induction of lipogenic enzymes in isolated hepatocytes. As shown in Figure 1A, incubation of hepatocytes isolated from 3 day starved rats with inducing medium (serum-free DMEA containing 27 mM glucose, 42 mU/ml insulin, and 1 μM dexamethasone) resulted in a 13-fold increase in G6PDH activity over three days in culture, a 4-fold increase above activity of cells incubated for three days in control medium. Control medium was serum-free DMEA which contained 5.6 mM glucose. 6PGDH activity of "induced" cells dropped 30% in the first and second days of culture then returned to the level present at the start of the culture by the third day (Figure 1B). Malic enzyme activity of "induced" cells remained constant or decreased slightly for two days, then increased 2-fold by the

Figure 1. Time Course Of Induction Of Lipogenic Enzymes In Primary Cultures Of Rat Hepatocytes. Male Holtzman rats weighing 200-220 g were starved for 3 days, ether anesthetized, and livers perfused with medium containing collagenase to isolate hepatocytes. Cells were plated onto tissue culture dishes which were coated with FBS and washed 5X with water. Culture medium was serum-free DMEA. For each dish, 3×10^6 cells were plated in 3 ml DMEA containing various metabolite and hormone additions and incubated at 37°C in a humidified, 5% CO_2/95% air environment. "Control" medium was DMEA and "induced" medium was DMEA supplemented with final concentrations of 27.3 mM glucose, 42 mU/ml insulin, and 1 μM dexamethasone. Values are means ± S.D. for three dishes.

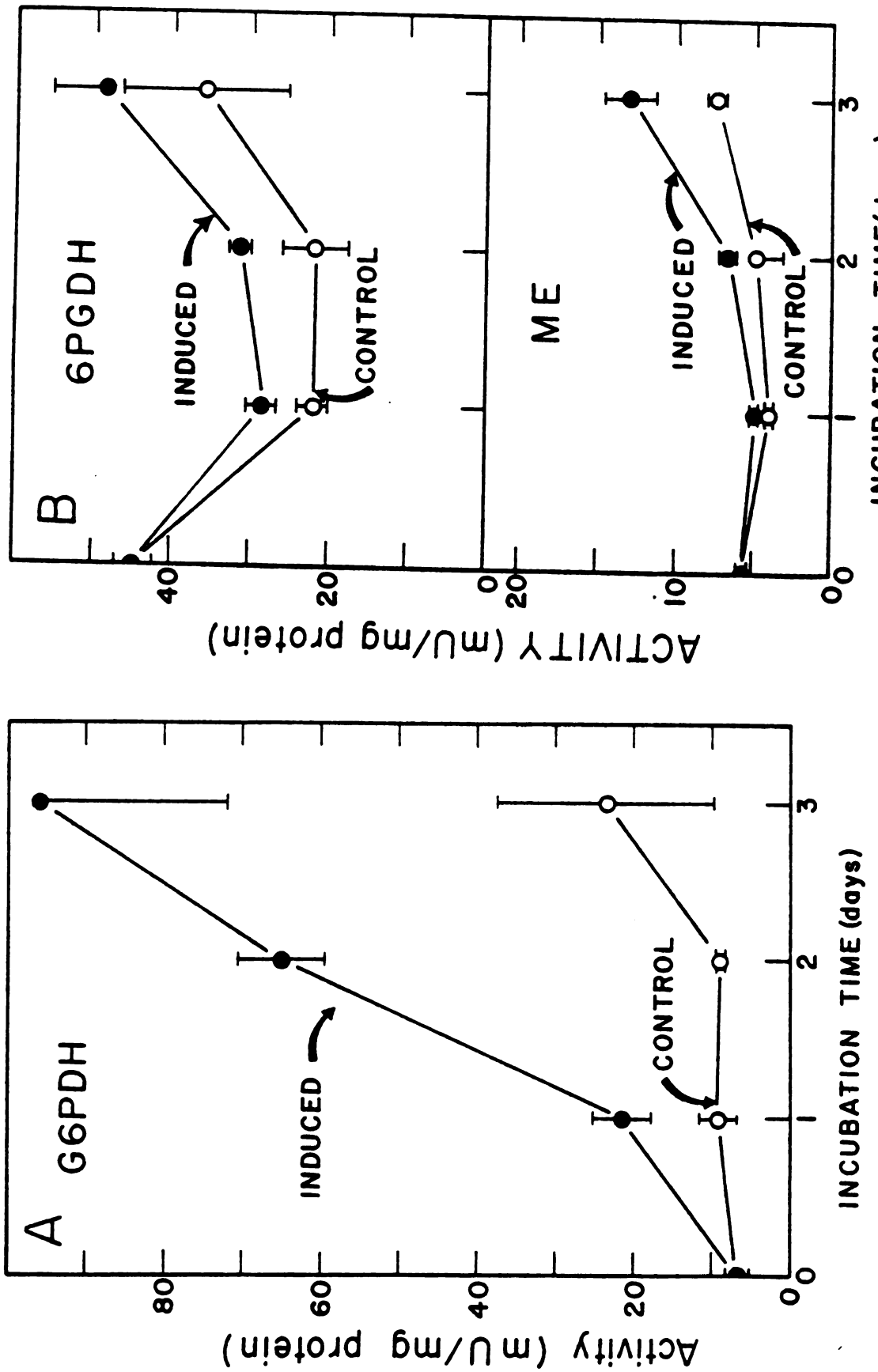

Figure 1. Time Course Of Induction Of Lipogenic Enzymes In Primary Cultures Of Rat Hepatocytes.

third day (Figure 1B). For both 6PGDH and ME, activities of control and induced cells were parallel, the induced state having slightly higher activities.

The responsiveness of the selected hepatocyte lipogenesis enzymes to varying amounts of glucose, insulin, and dexamethasone is shown in Figures 2-4. The specific activities of G6PDH, 6PGDH, and ME in hepatocytes incubated for 0 and 48 hours in control medium lacking glucose, insulin, and dexamethasone and 48 hours in medium containing 42 mU/ml insulin and 1μM dexamethasone with varying amounts of glucose are shown in Figure 2. In control medium, hepatocyte G6PDH activity increased 3.3-fold during 48 hours in culture. Cells incubated with insulin and dexamethasone, in the absence of glucose, had 11.4 and 3.5-fold elevated G6PDH activity above cells incubated 0 and 48 hours in control medium. Addition of glucose up to 22 mM had no further stimulating effect on the induction of G6PDH by insulin and dexamethasone. 6PGDH and ME activities of hepatocytes incubated in control medium dropped during 48 hours in culture and for 6PGDH but not ME, could be restored to 0 hr levels by addition of insulin and dexamethasone. As in the case for G6PDH activity, addition of glucose had no effect on 6PGDH and ME activities in hepatocytes incubated with insulin and dexamethasone.

Optimal concentrations of insulin and dexamethasone at a glucose concentration of 27.3 mM for the induction of G6PDH in hepatocytes from 3 day starved rats were 40 to 160 mU/ml and 1 to 10 μM respectively as shown in Figures 3 and 4. The degree to which glucose and hormones interact to affect lipogenesis enzyme induction in hepatocytes from starved rats was examined using optimal inducing concentrations of insulin (42 mU/ml) and dexamethasone (1 μM); 27.3 mM glucose and 15 μM T_3 in

Figure 2. Glucose Dose-Response Curve. Hepatocytes were prepared, cultured, and analyzed as described in Figure 1. In this experiment, incubation medium was a modified DMEA which was glucose-free. "No additions" refers to the absence of added insulin and dexamethasone. Glucose concentration varied as indicated and insulin and dexamethasone concentrations, where added, were 42 mU/ml and 1 μM respectively (final concentrations). Values are means ± S.D. for 3 dishes.

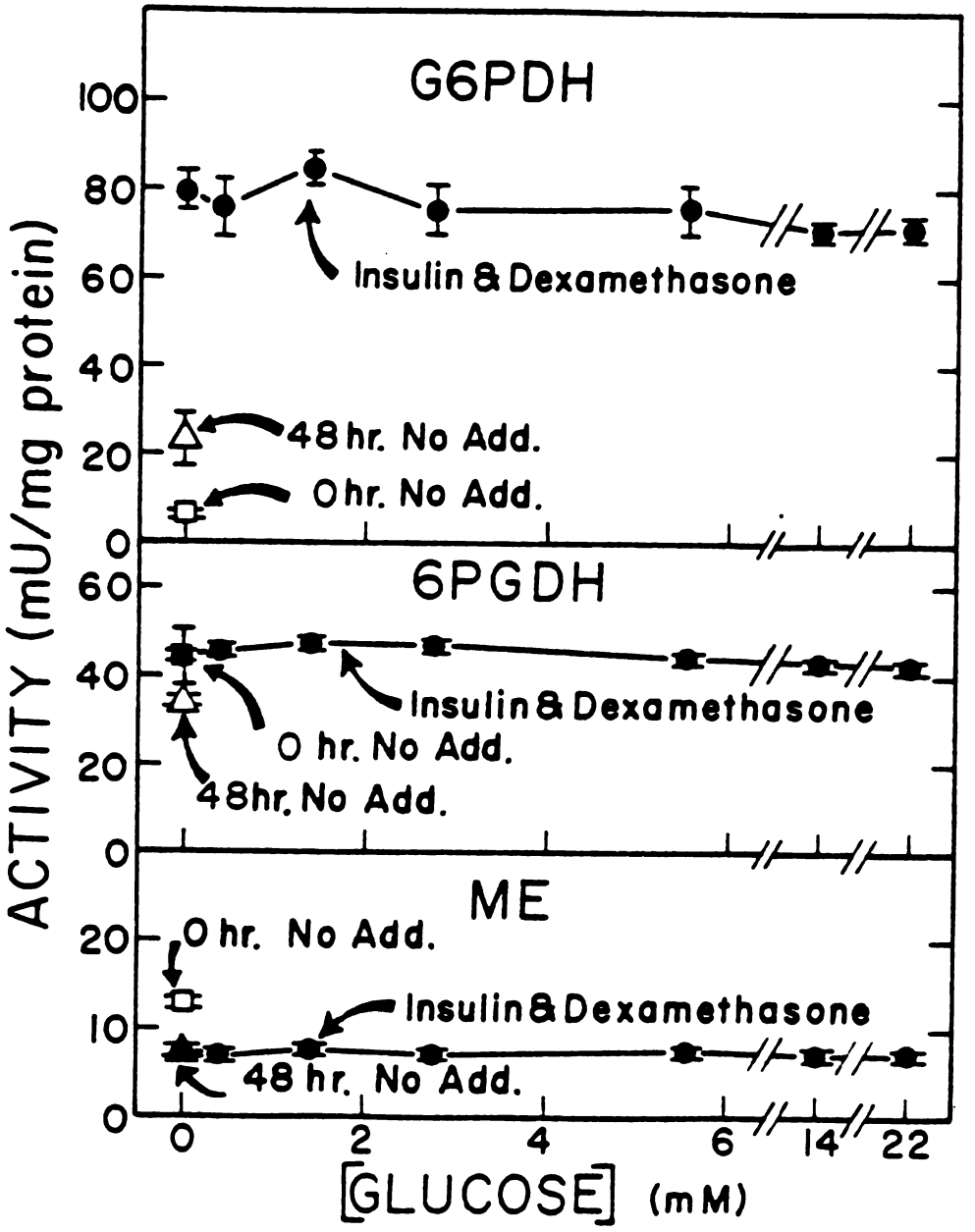

Figure 2. Glucose Dose-Response Curve.

Figure 3. Insulin Dose-Response Curve. Hepatocytes were prepared, cultured, and analyzed as described in Figure 1. In this experiment, incubation medium was DMEA. "No additions" refers to the absence of added glucose and dexamethasone. Insulin concentration varied as indicated and glucose and dexamethasone concentrations, where added, were 27.3 mM and 1 μM respectively (final concentrations). Values are means ± S.D. for 3 or 4 dishes or single determinations.

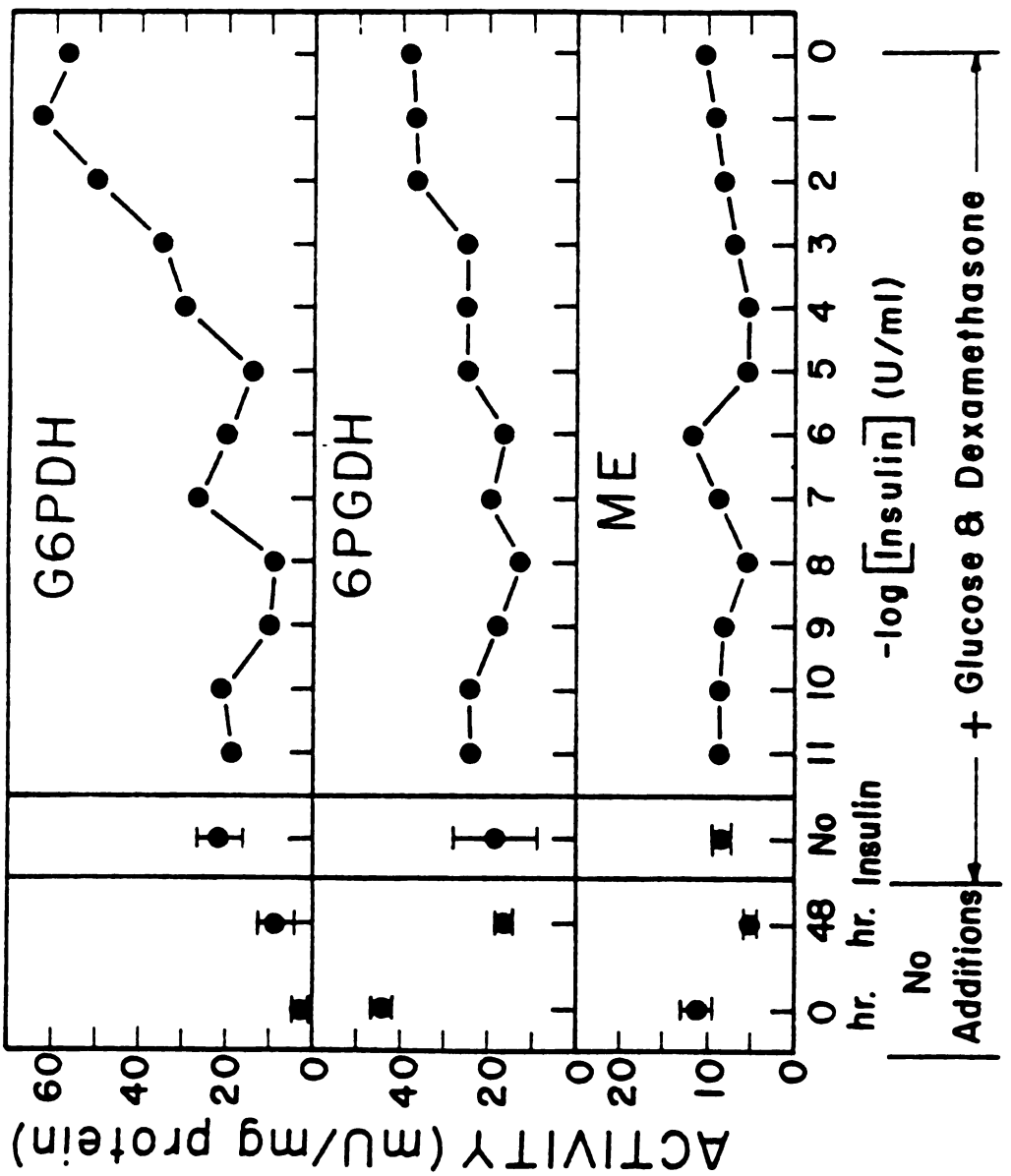

Figure 3. Insulin Dose-Response Curve.

Figure 4. Dexamethasone Dose-Response Curve. Hepatocytes were prepared, cultured, and analyzed as described in Figure 1. In this experiment, incubation medium was DMEA. "No additions" refers to the absence of added glucose and insulin. Dexamethasone concentrations varied as indicated and glucose and insulin concentrations, where added, were 27.3 mM and 42 mU/ml respectively (final concentrations). Values are means ± S.D. for 3 or 4 dishes or single determinations.

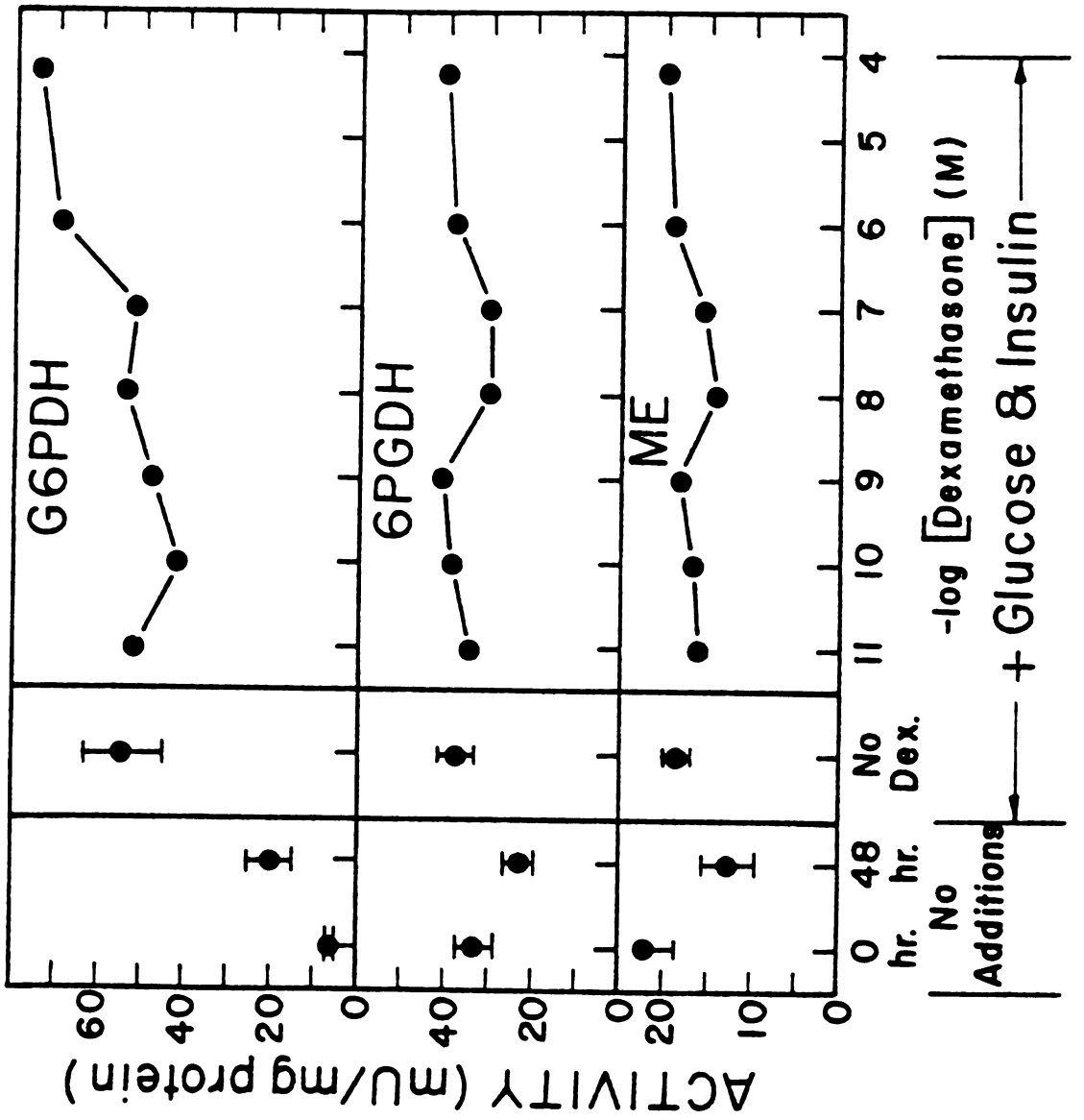

Figure 4. Dexamethasone Dose-Response Curve.

all possible combinations. The results are shown in Tables IV and V. Control G6PDH activity increased 2-fold during the 48 hour incubation. Addition of insulin or dexamethasone to the incubation medium led to a further 2-fold increase while glucose and T_3 had no significant effect on G6PDH activity. Insulin and dexamethasone together had a synergistic effect (3.7 fold increase) on G6PDH activity. The presence of T_3 (Table V) blunted, but did not totally inhibit, the insulin, dexamethasone or insulin + dexamethasone stimulated increase in G6PDH activity. Control 6PGDH activity decreased slightly during the 48 hour incubation. Insulin or glucose + insulin addition resulted in a slight but significant elevation in 6PGDH activity above 0 hour activity while dexamethasone or T_3 prevented this effect. Control ME activity dropped significantly during the 48 hour incubation. No hormone treatment was able to restore ME activity to starvation level.

A series of experiments (data not shown) was performed to determine the cause of the return of G6PDH activity to near normal values during 48 hours of incubation in control medium. The factors examined were as follows (in each case the first condition in parentheses is the usual control condition): medium pH (7.6 vs 7.0), cell density of plating (3.0×10^6 vs 4.5×10^6), tissue culture dish preparation (FBS-coated vs calf serum coated vs uncoated) frequency of medium change (once vs three times in 48 hours), and concentration of glucose (5.6 mM vs 0 to 22 mM), and a reexamination of assay linearity on a starved rat hepatocyte preparation over a protein concentration of 0.006 to 0.120 mg/ml assay mixture. None of these variations of experimental conditions provided an explanation for the increase in G6PDH activity in control incubations.

Table IV. Induction Of Lipogenic Enzymes In Primary Cultures Of Rat Hepatocytes. Hepatocytes were prepared, cultured, and analyzed as described in Figure 1. In this experiment, additions to DMEA, in final concentrations, are indicated in parentheses. Values are means ± S.D. for three dishes. Means not having a common superscript are significantly different as determined by analysis of variance, least significant difference test, $p < 0.05$.

Table IV. Induction of Lipogenic Enzymes In Primary Cultures of Rat Hepatocytes.

Treatment		Enzyme Activity (mU/mg protein)		
Time (hours)	Addition to Medium	G6PDH	6PGDH	ME
0	none	14.69 ± 1.00[a]	53.85 ± 3.87[c]	12.76 ± 0.74[a]
48	none	29.66 ± 10.58[b]	43.41 ± 7.29[b]	7.88 ± 1.15[c,d]
48	glucose (27.3 mM)	39.02 ± 3.66[b]	43.94 ± 2.70[b]	8.69 ± 0.80[d,e]
48	insulin (42 mU/ml)	56.84 ± 8.06[c]	67.75 ± 1.22[d]	9.54 ± 1.09[e]
48	dexamethasone (1 μM)	57.58 ± 9.79[c]	32.35 ± 3.97[a]	6.12 ± 0.59[b]
48	glucose + insulin	53.53 ± 6.58[c]	77.13 ± 2.76[e]	8.95 ± 0.35[d,e]
48	glucose + dexamethasone	66.67 ± 10.78[c]	42.69 ± 3.00[b]	6.62 ± 0.66[b,c]
48	insulin + dexamethasone	111.15 ± 10.48[e]	66.63 ± 4.83[d]	9.12 ± 0.64[d,e]
48	glucose + insulin + dexamethasone	87.03 ± 3.76[d]	52.11 ± 0.82[c]	7.94 ± 0.49[c,d]

Table V. Effect of Triiodothyronine on the Induction of Lipogenic Enzymes in Primary Cultures of Rat Hepatocytes. Hepatocytes were prepared, cultured, and analyzed as described in Figure 1. Values in parentheses are the percentage change from comparable values obtained from cells incubated in the absence of triiodothytronine (T_3) shown in Table IV. Values are means ± S.D. for three dishes. Means not having a common superscript are significantly different as determined by analysis of variance, least significant difference test, $p < 0.05$.

Table V. Effect of Triiodothyronine on the Induction of Lipogenic Enzymes in Primary Cultures of Rat Hepatocytes.

Treatment		Enzyme Activity (mU/mg protein)							
Time (hours)	Addition to Medium	G6PDH	(%)[a]	6PGDH	(%)[a]	ME	(%)[a]
48	T_3 (15 μM)	36.08 ± 4.10 [a,b,c]	(22)	45.68 ± 2.86 [b,c]	(5)	8.92 ± 0.91 [b,c,d]	(13)
48	T_3 + glucose (27.3 mM)	30.19 ± 3.75 [a,b]	(-23)	55.49 ± 3.21 [c,d]	(26)	9.37 ± 0.55 [c,d]	(8)
48	T_3 + insulin (42 mU/ml)	49.44 ± 8.70 [c,d]	(-13)	65.53 ± 4.92 [d]	(-3)	9.77 ± 0.39 [d]	(2)
48	T_3 + dexamethasone (1 μM)	25.91 ± 2.60 [a]	(-55)	28.39 ± 0.59 [a]	(-11)	6.41 ± 0.37 [a]	(5)
48	T_3 + glucose + insulin	41.97 ± 11.35 [b,c]	(-22)	66.87 ± 11.18 [d]	(-13)	8.55 ± 1.72 [b,c,d]	(-4)
48	T_3 + glucose + dexamethasone	32.57 ± 2.61 [a,b]	(-51)	37.19 ± 1.48 [a,b]	(-13)	7.09 ± 0.46 [a,b]	(7)
48	T_3 + insulin + dexamethasone	63.91 ± 6.65 [c]	(-43)	50.35 ± 5.16 [c]	(-24)	7.66 ± 0.81 [a,b,c]	(-16)
48	T_3 + glucose + insulin + dexamethasone	58.80 ± 3.34 [d,e]	(-32)	48.52 ± 3.52 [b,c]	(-7)	9.63 ± 0.61 [d]	(21)

Although cell growth is reported by most authors not to occur in primary cultures of adult rat hepatocytes, there are reports that, especially during prolonged incubation, cell division can occur (see 36). Microscopic examination of cells in culture revealed a progressive increase in confluence of cells suggesting the possibility of cell division. This possibility was particularly important since the induction of G6PDH could be linked to a demand for ribose needed for DNA synthesis. Also because insulin is known to have growth promoting characteristics in other types of mammalian cells (46) it was essential to examine whether insulin was mitogenic in these cells. Aphidicolin, at 5 μg/ml, a concentration sufficient to block DNA synthesis in dividing cultures of Hela (47) and CHO cells (personal communication from Dr. John A. Boezi), did not block the insulin and dexamethasone mediated induction of G6PDH nor was there an effect on total DNA content (Table VI). Thus, induction occurred in these cultures of hepatocytes in a manner which was independent of DNA synthesis.

Table VI. Effect of Aphidicolin on DNA Synthesis and Induction of Lipogenic Enzymes in Primary Cultures of Rat Hepatocytes.

Treatment			Enzyme Activity (mU/mg protein)		
Incubation time (hours)	Aphidicolin (5 µg/ml)	DNA (µg/plate)	G6PDH	6PGDH	ME
0	-	26.4	5.33 ± 1.74[a]	43.99 ± 4.48[b]	5.70 ± 0.71[c]
24	-	18.9	18.80 ± 1.22[b]	29.10 ± 1.02[a]	3.89 ± 0.85[a,b]
24	+	14.8	15.08 ± 1.04[b]	27.50 ± 1.55[a]	2.68 ± 0.47[a]
48	-	15.9	41.08 ± 13.75[c]	24.50 ± 2.96[a]	4.19 ± 0.64[b]
48	+	13.9	47.22 ± 5.14[c]	26.69 ± 1.23[a]	4.39 ± 0.28[b]

[a]Hepatocytes were prepared, cultured, and analyzed as described in Figure 1. In this experiment cells were incubated under inducing conditions in the presence or absence of aphidicolin as indicated. Values are means ± S.D. for three dishes. Means not having a common superscript are significantly different as determined by analysis of variance, least significant difference test, $p < 0.05$.

DISCUSSION

The amount of dietary carbohydrate consumed during refeeding was considered by some authors (2-7) to be the primary signal for the induction of hepatic lipogenesis enzymes. Gozukara et al. (4) have shown a significant linear correlation between the kcal of dietary carbohydrate consumed/day/100 g body weight and the immunochemically detected rate of synthesis of G6PDH in starved-refed rats. In another study from the same laboratory (2), rats were starved then refed a 60% fructose containing diet. Following refeeding, it was observed that the induction of G6PDH was esentially the same as that achieved by refeeding a 60% glucose diet where an insulin response is known to occur. The authors concluded that insulin must be ruled out as an intermediary signal between diet consumption and induction of G6PDH based on a report that fructose failed to elicit an insulin release from rabbit pancreas (48). However, Sugawa-Katayama and Morita (49) found that rats starved and refed a 69% fructose diet responded with a significant increase in serum immunodetectable insulin. If dietary glucose is the primary signal for the induction of lipogenic enzymes, then one would expect rats depleted of insulin to be as capable of inducing G6PDH as intact rats. The results presented here (Table I) do not support this view. Streptozotocin-diabetic rats given the same starvation-refeeding regimen as normal rats are significantly impaired in their ability to induce G6PDH, 6PGDH and ME. The induction of G6PDH, 6PGDH, and ME in diabetic rats was reduced to 10, 22, and 13% respectively of that in intact starved-refed rats. These results confirm those of Weber and Convery (16) and extend them to include ME. In addition, the results presented here demonstrate that this impaired

ability of a diabetic starved/refed rat to induce lipogenic enzymes to the same extent as a normal starved/refed rat is not due to a dimimished diet consumption by the refed diabetic rat (Table III). In fact, diabetic refed rats consume more diet/100 g body weight than normal refed rats (Table III) and should, if dietary glucose were the major stimulus, show enhanced induction of the lipogenic enzymes. The observation presented here that isolated hepatocytes incubated with insulin and dexamethasone in the absence of glucose are able to induce G6PDH (Figure 2) also supports the conclusion that glucose is not required for the induction of G6PDH.

Another opinion (16-18,21) is that insulin plays an important role in the induction of lipogenic enzymes by starvation-refeeding. In the present study, injection of starved diabetic rats with insulin during the refeeding period resulted in the significant induction of the three hepatic lipogenic enzymes examined (Table I), thus extending the work of Weber and Convery (16) to include ME. As shown in Table III, insulin supplementation to starved diabetic rats during refeeding lowered the diet consumption/100 g body weight compared with non-insulin treated starved/refed diabetic rats. Thus the observed induction of the lipogenic enzymes in insulin supplemented starved-refed diabetic rats cannot be attributed to an increased diet consumption. The simplest interpretation of these results is that the injected insulin acted directly on the liver to increase the lipogenic enzyme activities, however it was still possible that insulin was acting either in concert with some other <u>in vivo</u> factors or indirectly by stimulation of another organ to release an inducing factor(s). In cultured hepatocytes from 3 day starved rats, it was possible to demonstrate the induction of G6PDH by added insulin in

the absence of glucose (Figure 2). The addition of up to 22 mM glucose did not alter the induction process. The consistent 2-fold increase in G6PDH activity in 48 hour cultures of hepatocytes from 3 day starved rats to levels found in hepatocytes from normal unstarved rats could be explained as the result of the translation of a pool of stable G6PDH mRNA. Szepesi et al. (50) have found an 8-azaguanine resistant increase in G6PDH activity in starved-refed rats and suggest that this is due to the translation of a pool of stable G6PDH mRNA which is not degraded during starvation. If such a pool existed in hepatocytes from 3-day starved rats, then replenishment of amino acids, in the absence of further inducing signals, could lead to a return of G6PDH activity to pre-starvation levels.

The stimulatory effect of dexamethasone on the induction of G6PDH in primary cultures of rat hepatocytes (Table IV) is consistent with the observations, in vivo, of Berdanier et al. (23-26). Optimal amounts of insulin and dexamethasone, in combination induced G6DPH to a greater extent than either alone. The additive nature suggests that these agents are acting by different mechanisms. Recently, Holten et al. (28,30) reported that the dietary and hormonal stimulated increase in G6PDH mRNA was not sufficient to account for the observed magnitude of increase in G6PDH synthesis (29). In this regard, Peraino (51) has suggested that glucocorticoids may exert a permissive role in the induction of G6PDH and glucokinase, in vivo, by making more intracellular amino acids available for translation.

Unlike the in vivo response to injected insulin (Table I), 6PGDH does not appear to be induced to a great extent by insulin in vitro (Figure 3 and Table IV). In dose-dependent fashion, insulin appears to

prevent a decrease in enzyme activity during culture. ME also does not respond to insulin addition *in vitro* (Figure 3 and Table IV) to the extent observed after insulin administration *in vivo* (Table I). Recent results of Nakayama and Holten indicate that a greater induction of 6PGDH by insulin occurred after incubation of hepatocytes for 7 days (27). Dexamethasone did not improve the response of 6PGDH or ME to insulin in the studies reported here.

Thyroid hormone participation in the induction of G6PDH, 6PGDH, and ME was examined because of the well documented effects of T_3 on the induction of ME in the rat *in vivo* (33) and in chick embryo liver cells in culture (52). Also, the recent reports of Spence *et al.* which showed that hepatocytes obtained from thyroidectomized rats did not induce ATP-citrate lyase (53) or glucokinase (54) in response to insulin and dexamethasone unless pre-treated with T_3 indicated the possible importance of thyroid hormones for the induction of other lipogenic enzymes. Addition of T_3 to cultures of hepatocytes incubated as described in Table V indicated that this hormone was slightly inhibitory but does not prevent the insulin and dexamethasone stimulated induction of G6PDH. The activity of 6PGDH was also lower in cells incubated 48 hours in the presence of T_3 and the various combinations of glucose, insulin, and dexamethasone shown in Table V while cellular ME activity was elevated only slightly by such treatment.

The lack of a significant T_3 effect on ME activity in hepatocytes from 3 day starved rats could be explained by the relatively long $t_{1/2}$ of ME in response to T_3 (4 days) (55) and the decrease in the nuclear T_3 binding capacity during starvation (56).

In conclusion, the *in vivo* and *in vitro* results presented here indicate that glucose, either consumed by starved diabetic rats or added to the culture medium of hepatocytes obtained from 3 day-starved rats alone is not sufficient to elicit an induction of G6PDH, 6PGDH, or ME. Insulin, when injected into starved diabetic rats during a period of refeeding, resulted in an induction of G6PDH, 6PGDH, and ME in a manner independent of dietary glucose consumption. In isolated hepatocytes, insulin, and dexamethasone, in the absence of medium glucose, acted separately or together to induce G6PDH. 6PGDH and ME activities were not greatly affected by these treatments. The mechanism by which insulin and dexamethasone induce rat liver G6PDH remains to be elucidated.

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SUMMARY

The development of automated fluorometric analyses for common biochemical determinations (Chapter I) may prove helpful to persons doing large numbers of samples from tissue culture materials.

The experiments performed in Chapters II and III were designed to determine if the model for lysosomes proposed by Szego (1,2) for estradiol action could be extended to include glucocorticoid stimulated induction of tyrosine aminotransferase in RH-35 cells and starvation-refeeding stimulated induction of lipogenesis enzymes in rat liver. The results presented here do not support this extension. One could argue that the disruptive nature of the cellular fractionation techniques used may have disturbed the physiological association of lysosomes with nuclei, however it should be noted that the nuclear purification procedure used was that of Szego and Seeler (3). Also the electron microscopic evidence failed to show any redistribution of lysosomes within the RH-35 cell after glucocorticoid stimulation at time points comparable to those chosen by Szego.

The observations of Schroeder *et al.* (4), that there is a transient liver lysosome fragility coincident with a shift from the starved to the refed state, was confirmed by the results of Chapter III. However, the failure of lysosome stabilization and labilization to alter expected changes in the induction of lipogenic enzymes would suggest that, while a transient fragility is occurring simultaneously with induction, it is not an integral part of the induction signal *per se*. It is possible that these fragility changes could reflect recently observed association of internalized insulin-receptor complexes with lysosomes (5,6). This

represents an exciting role for lysosomes as agents of either destruction or further processing of hormonal stimuli.

In the final Chapter, evidence is presented that insulin is required for the overshoot induction of G6PDH *in vitro* and *in vivo* and that glucose alone is neither sufficient nor necessary for induction of G6PDH. This is in direct disagreement with the previous results of Holten *et al.* (7,8). This discrepancy is discussed in detail in Chapter IV.

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APPENDIX

Table I. Comparison of Liver Total and Nuclear Cathepsin D and Liver Cytosolic Lipogenic Enzyme Activity between Genetically Obese Male Mice and their Lean Male Littermates.[a]

Animals	Age (days)	Cathepsin D: Total Liver Activity (U/liver)	Cathepsin D: Total Liver Activity (U/mg DNA)	Cathepsin D: Nuclear Activity (U/mg DNA)	Cytosolic Enzyme Activity (mU/mg protein): G6PDH	Cytosolic Enzyme Activity (mU/mg protein): 6PGDH
lean	61-78	3.27 ± 0.47 (12)	6.07 ± 2.02 (15)	5841 ± 2195 (15)	7.9 ± 1.2 (15)	27.5 ± 5.5 (15)
obese	61-76	6.33 ± 0.97*(12)	6.78 ± 2.43 (15)	9186 ± 3724*(15)	6.3 ± 2.5*(12)	57.5 ± 12.5*(15)
lean	152-158	2.03 ± 0.84 (13)	4.30 ± 0.95 (13)	3041 ± 970 (13)	12.0 ± 4.6 (13)	36.6 ± 7.3 (13)
obese	152-158	5.91 ± 2.32*(13)	4.71 ± 1.61 (13)	3202 ± 556 (13)	12.0 ± 3.6 (13)	69.8 ± 13.1*(13)

[a]Mice were the C57BL/6J - ob/ob strain and their normal homozygous or heterozygous littermates obtained from The Jackson Laboratory, Bar Harbor, ME. Cathepsin D activities were determined as described in the Methods section of Chapter II and dehydrogenase activities were determined as described in the Methods section of Chapter III. For total liver Cathepsin D activity: U/liver = cpm x 10^6/90 min/liver and U/mg total DNA = cpm x 10^5/90 min/mg DNA; for nuclear Cathepsin D activity, U/mg DNA = cpm/90 min/mg nuclear DNA. Unit G6PDH and 6PGDH activities are μmoles NADPH produced/min at 30°C. Values are means ± S.D. for the number of animals given in parentheses.

*Mean differs significantly ($P < 0.05$) from that of lean littermates. Statistics were analysis of variance, randomized complete block design with lean and obese as blocks.

Figure 1. Time course of Liver Glucokinase Activity during Starvation-Refeeding. Liver cytosol glucokinase activities were determined as described in Methods in Enzymology Vol IX p. 381-392, 1966. The starvation-refeeding regimen was as described in the Methods section of Chapter III. The dotted line was calculated from the equation:

$$[E_t] = [E_s] - ([E_s] - [E_0])\, e^{-kt},$$

where:

E_t = enzyme activity at a given time,
E_s = enzyme activity at the new steady state level,
E_0 = enzyme activity initially,
k = rate constant of change in activity, and
t = time, according to Gibson, D.M. et al. (1972) Adv. Enz. Reg. 10, 187-204.

Points are experimentally obtained data. $[E_s] = 37.3$ and $[E_0] = 5.23$ nmoles/min/mg protein. Using the equation:

$$t_{1/2} = \ln 2/k,$$

$k = 1.66$ days^{-1} during refeeding.

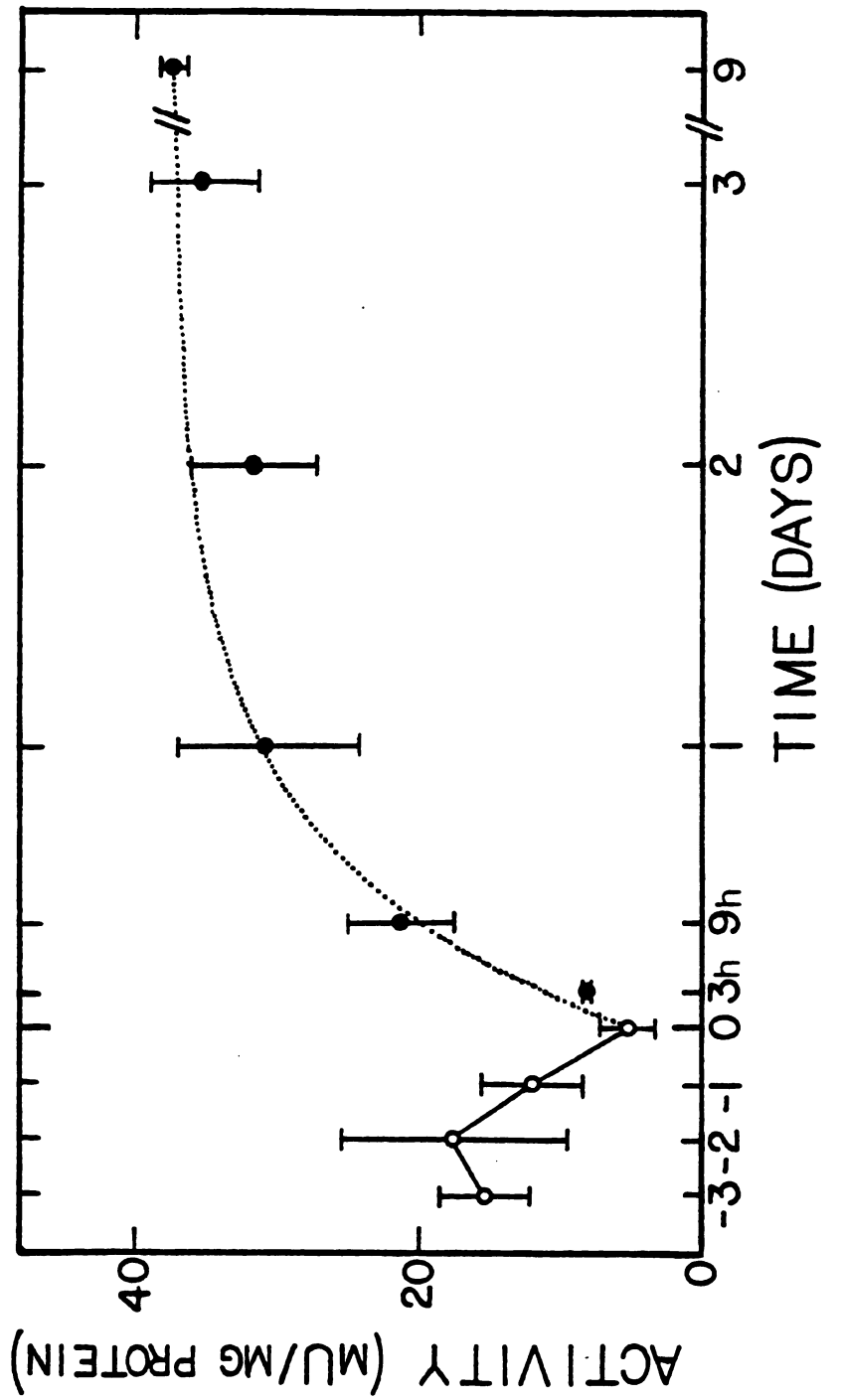

Figure 1. Time course of Liver Glucokinase Activity during Starvation-Refeeding.