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EARLY EFFECTS OF PHENOBARBITAL ON NUCLEAR-CYTOPLASMIC RELATIONSHIPS IN RAT LIVER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

John D. Young, B.S.

***

The Ohio State University
1976

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Publications and Abstracts


Young, J.D. and Gehring, P.J.: The Dose-Dependent Fate of 1,4-Dioxane in Male Rats. Toxicol. Appl. Pharmacol. 33: 183 Abs, 1975.

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INTRODUCTION

The treatment of animals with a variety of lipid soluble foreign compounds often results in the enhancement of liver microsomal drug metabolizing activities (Brown, et al., 1954). This enhancement has been called induction. The drug phenobarbital was added to the list of compounds which induce drug metabolizing activities by Remmer (1959) and is one of the most potent compounds with this property.

Chronic administration of phenobarbital to rats causes morphological changes in the hepatocyte including a massive proliferation of the smooth endoplasmic reticulum (Remmer and Merker, 1963), which is probably the major site of drug metabolizing enzymes (Fouts, 1961). Chronic phenobarbital treatment causes an increase in liver weight (1.5 fold) which is accompanied by an increase in microsomal protein, but not in mitochondrial protein (Orrenius, et al., 1965). Further studies by Orrenius, et al, have shown that the increases in microsomal protein include the content of cytochrome P-450 and the activities of aminopyrine demethylase and NADPH-cytochrome c reductase, while the activities of glucose-6-phosphatase, ATPase, and NADH-cytochrome c reductase are reduced. The enzyme activities which increase appear first in the rough surfaced membranes (3-6 hours) and then in
the smooth surfaced membranes (24 hours). These authors conclude that enzyme synthesis proceeds in the rough surfaced membranes and, after "saturation" of these membranes with enzyme, the ribosomes then become attached to the smooth surfaced membranes.

Shuster and Jick (1966) studied the turnover of microsomal protein in fed mice treated with phenobarbital and found evidence for both increased synthesis and decreased breakdown. However, Greim (1970) observed that the nutritional state of the animal is an important consideration in assessing data on induction of microsomal protein. Using $^{14}\text{C-\delta-aminolevulinic}$ acid to label the heme moieties of rat liver microsomal cytochrome P-450, Greim found that in fasted rats phenobarbital caused an increase in cytochrome P-450 due to increased synthesis and decreased degradation. In fed rats phenobarbital caused an increase in cytochrome P-450 due to increased synthesis, but not due to decreased degradation.

Further evidence for de novo synthesis of microsomal protein due to phenobarbital treatment comes from the use of inhibitors of protein synthesis such as actinomycin D, puromycin (Orrenius, et al., 1965), and ethionine (Kato, et al., 1965) to block this response. Phenobarbital stimulates the incorporation of radioactive precursors into microsomal protein in vivo (Kato, et al., 1965) and in vitro (Kato, et al., 1966).
The specific activity of microsomal RNA was found to increase at 12 and 18 hours in rats treated with phenobarbital and \(^{14}\text{C}\)-orotic acid, an RNA precursor (McCauley and Couri, 1971). Most of the increase in RNA was found in membrane bound ribosomes. Cycloheximide, an inhibitor of protein synthesis, reduced the increases in specific activity of RNA in both bound and free ribosomes. These data suggest that phenobarbital caused an increase in either the number or affinity of binding sites for ribosomes on the endoplasmic reticulum, and support the hypothesis that a protein factor which is involved in transport of ribosomal RNA from the nucleus (McNamara, et al, 1973) may be activated or induced by phenobarbital.

Other attempts to explain the increase in microsomal RNA after phenobarbital treatment include the inhibition of microsomal ribonuclease (Mycek, 1971), which was correlated in time with the increase in demethylase activity and persisted in the absence of the pituitary or adrenal glands (Louis-Ferdinand and Fuller, 1972). The latter authors suggest that the ribonuclease inhibition was due to the presence of a cytoplasmic inhibitor and was not due to a direct action of the drug on the enzyme.

Since phenobarbital treatment causes a proliferation of the smooth endoplasmic reticulum in addition to the effects on protein and RNA content, studies have been conducted on the lipid portion of the microsomes. Repeated
phenobarbital administration causes an increase in the amount of liver microsomal lipid phosphorus which parallels the increase in demethylase activity, but after cessation of treatment the decay in lipid phosphorus content is much slower than the decay of enzyme activity (Orrenius, 1968). The injection of actinomycin D with phenobarbital diminished but did not abolish the increase in $^{32}P$ incorporation into microsomal phospholipid compared to control animals (Orrenius, 1966). These data indicate that the mechanism for induction of membrane phospholipid may be different from the mechanism for induction of microsomal drug metabolizing enzymes, although both processes as well as the increase in specific activity in ribosomal RNA (McCauley and Couri, 1971) seem to be dependent on protein synthesis.

A common feature of all compounds examined is their binding to the liver microsomal fraction. The binding of phenobarbital to liver microsomes has been studied in vivo (Ernster and Orrenius, 1965) and in vitro (Orrenius and Ernster, 1967). The in vitro binding was specific for liver microsomes, stoichiometric with cytochrome P-450 content, inhibited by CO, and enhanced by prior phenobarbital treatment. The probable binding site for phenobarbital is therefore cytochrome P-450, which also binds other drugs and steroid hormones. The in vivo treatment of rats with $^{14}$C-phenobarbital resulted in a maximum drug binding to microsomes at 3 hours, and about 1.5 moles of drug bound per mole of microsomal cytochrome P-450.
Since phenobarbital binds to liver microsomes and may displace the binding of endogenous steroid hormones, it is possible that the nuclear events which occur after drug treatment are due to displaced steroid hormones. This possibility was studied by Orrenius, et al., (1968) who followed the effects of phenobarbital on adrenalectomized-castrated male rats. The content of cytochrome P-450 and the rate of drug metabolism decreased 75% in the steroid deficient animals, and phenobarbital treatment returned both to levels slightly above controls. Phenobarbital plus testosterone treatment resulted in induced levels of cytochrome P-450 and enzyme activities which were equal to levels in non-operated, phenobarbital treated animals. The authors concluded that steroid hormones are involved in both the maintenance of normal drug metabolizing activity in liver microsomes and in the drug induced increase of enzyme activity.

The involvement of steroid hormones in the maintenance of normal drug metabolizing activity observed by Orrenius, et al., (1968) suggests that the circadian rhythmicity in the production of steroids should be reflected in a similar circadian rhythmicity in the activity of drug metabolizing enzymes. Recently, Chedid and Nair (1972) demonstrated a diurnal rhythm for the activity of hexobarbital oxidase, a microsomal enzyme, and for the morphology of the endoplasmic reticulum as well. These authors also demonstrated a diurnal rhythm for the inducibility of hexobarbital oxidase by phenobarbital. The mortality of a high single dose of
phenobarbital to rats was found to be strikingly dependent on the time of day the dose was administered (Muller, 1971), demonstrating that the circadian rhythmicity of drug metabolism has a profound effect on the detoxification of foreign compounds.

The involvement of the nucleus in circadian changes in drug metabolism was supported by the observations of Glasser and Spelsberg (1972) who demonstrated a circadian rhythmicity in the activities of DNA dependent RNA polymerases which synthesize both messenger-like and ribosomal-like RNA. Using the terminology of Roeder and Rutter (1970), RNA polymerase I is located in the nucleolus and produces ribosomal-like RNA. The nucleolar enzyme activity is insensitive to the inhibitor α-amanitin, produces an RNA with a high content of the bases guanosine and cytosine, and is stimulated maximally in vitro by magnesium ions and low salt concentration. The nuclear enzyme, RNA polymerase II, produces messenger-like RNA. The nuclear enzyme activity is sensitive to the inhibitor α-amanitin, produces an RNA rich in adenosine and uracil, and is stimulated maximally in vitro by manganese ions and a high salt concentration.

Glasser and Spelsberg (1972) observed nearly a reciprocal relationship with time between the activities of RNA polymerase I and II in rat liver nuclei. With a 12-hour light cycle from 7:00 a.m. until 7:00 p.m., RNA polymerase I activity was maximal in the early light period and decreased in the later light period to half the maximum values. The activities of
rat liver RNA polymerase I and II were shown to be stimulated by phenobarbital treatment by Gelboin, et al., 1967 and Orrenius, et al., 1968. Although the relationship between enzyme induction by phenobarbital and its stimulation of nuclear and nucleolar RNA polymerases is not clear, it is interesting to speculate that phenobarbital may mediate its anabolic effects on the liver through an action in the nucleus.

Speculation that phenobarbital may mediate its effects on the liver through a more direct effect on the nucleus is not new. Conney (1967) in reviewing the literature on microsomal enzyme induction, suggested that agents which produce more liver microsomal enzyme may act on the nucleus in one or more of the following ways: 1) by increasing the synthesis of messenger RNA; 2) by interacting with repressors synthesized by a regulator gene, and; 3) by interacting with other regulators of nuclear function, such as histones. Conney also suggested mechanisms for induction which are indirectly related to nuclear function: 1) an interaction of the drug with the endoplasmic reticulum to increase translation of messenger RNA; 2) an interaction of the drug with microsomal enzymes to decrease the degradation of these enzymes, and; 3) an interaction of the drug to prevent feedback inhibition of enzyme synthesis.

Much is known about the effects of phenobarbital in the rat liver. However, the relationships of these effects to the mechanism of enzyme induction is not clear. Since
enzyme induction may have profound effects on the detoxification of foreign compounds and on the activation of chemical carcinogens (Peraino, et al., 1973), a knowledge of the mechanism by which many chemicals, including phenobarbital, induce microsomal enzyme activity has obvious utility.

**Working Hypothesis**

Based on present knowledge of events which occur in rat liver after phenobarbital treatment, the nucleus is clearly involved. However, it is difficult to determine if the nuclear events are due to a primary action of the drug or are secondary events due to changes evoked in the cell cytoplasm or on membranes prior to activation of the nucleus.

The working hypothesis tested in this study was based on an analogy of the action of steroid hormones in activation of specific genomes within a cell nucleus causing an increased synthesis of protein (Gorski, et al., 1973). Since the action of phenobarbital is specific, in that not all microsomal enzymes are induced, it was assumed that the drug exerts its action by combining with a specific receptor. This receptor may be a protein, as is the case for estrogen (Gorski, et al., 1973), or other component of the cell such as RNA, DNA, or membrane). One requirement for a phenobarbital receptor is that it must be present before administration of the drug, i.e., it may not be a product of induction.

A second assumption made was that the initial event in the liver cell must occur within a short time after administration of the drug. If the parent compound is the active
species, the initial event must occur as soon as the compound is distributed to the liver; if a metabolite is the active species, a short lag time may be required. Thus, studies were designed primarily to observe changes in the liver cell nucleus within 1 hour after drug administration. Other investigators have observed a stimulation of nuclear RNA polymerase activity 8 or more hours after drug treatment (Gelboin, et al., 1967 and Orrenius, et al., 1968), but the earliest time investigated was 2 hours.

The rapid growth produced in rat liver after phenobarbital is in many ways similar to the rapid growth produced as a result of partial hepatectomy (Campbell, et al., 1964). One of the most striking changes observed following partial hepatectomy is the rapid increase in the activity of ornithine decarboxylase (Russell, 1973). The soluble enzyme ornithine decarboxylase catalyzes the decarboxylation of ornithine to putrescine, one of a family of polyamines which have been linked to rapid growth and to the synthesis of nucleic acids (Tabor and Tabor, 1964). It has been demonstrated that phenobarbital treatment of rats for one week causes a 2.5 fold increase in the liver content of spermidine, a polyamine produced in the liver from putrescine (Vacha and Seifert, 1972). The concentration of various polyamines in rat liver has not been determined at early times after a single dose of phenobarbital. Therefore, the effect of phenobarbital treatment on rat liver polyamines as well as ornithine decarboxylase activity was investigated in this work.
The overall objective of this work was to determine the very early biochemical effects of a single dose of phenobarbital to rats on the function of the hepatocyte nucleus and to elucidate a possible mechanism by which these changes might occur.
METHODS

Animals

Male albino Wistar rats weighing between 140 and 250 g were obtained from Carworth Farms, Indiana. They were housed in an environment with the temperature and humidity controlled at 22° and 50%, respectively, and a 12 hour light-dark cycle beginning at 7:00 a.m. Free access was given to food, Purina Lab Chow, and water. Rats were sacrificed by the indicated techniques between 10:00 a.m. and noon.

Sodium phenobarbital was dissolved in sterile saline (Travenol) to make a solution approximately 40 mg/ml. The dose, 80 mg/kg, was administered intraperitoneally to the rats from a 1 ml syringe equipped with a 22 gauge needle. At this dose the rats were sedated but did not lose their righting reflex.

Radiolabeled Dose

Either $^{14}$C or $^3$H-labeled phenobarbital, used as radiotracers, were obtained from New England Nuclear Corp., Boston. Initial experiments using 5-ethyl-5-phenylbarbituric-$^{14}$C acid, specific activity 3.1 mCi/m mole, indicated that a greater specific activity was necessary to detect protein binding of small amounts of the compound. Therefore, 5-ethyl-5-phenyl-barbituric-4,5-$^3$H acid, specific
activity 10.3 Ci/mmole, was used subsequently. Thus, the $^3$H-labeled phenobarbital had a specific activity over 3300 times greater than the $^{14}$C-labeled compound, and was used without dilution with unlabeled phenobarbital unless such a dilution was indicated.

The radiochemical purity of each compound was reported by New England Nuclear to be greater than 99%. The reported radiochemical purity was verified by thin layer chromatography of an aliquot on Silica Gel $F_{254}$ plates developed with benzene-ethyl ether (9:1). Following development of the chromatograph, 1 cm squares were scraped into scintillation vials and the $^{14}$C or $^3$H activity determined by liquid scintillation counting in a Packard Tri-Carb spectrometer (Packard Instruments, Downers Grove, Ill.). A single spot of radioactivity with an $R_f$ of 0.6 which corresponded to the $R_f$ of authentic unlabeled phenobarbital was found.

Preparation of Rat Liver Nuclei—Method I

Nuclei prepared by Method I were used to assay for RNA polymerase I or II. This procedure was described by Spelsberg et al, 1974, to yield nuclei which retain maximum RNA polymerase activity, and is summarized here with modifications.

Rats were killed by decapitation between 10:00 a.m. and noon. Livers were quickly removed, rinsed by dipping into cold saline, blotted and weighed. Subsequent steps were performed with buffers maintained at 4° in ice buckets.
Each liver was homogenized in 20 volumes of a buffer composed of 0.05 M tris-HCl, 0.025 M KCl, and 0.05 M MgCl$_2$ (TKM buffer) and sucrose to make a 2.0 M solution. This solution (Solution A) was adjusted to pH 7.5. An aliquot of the homogenate equivalent to 2 g of liver (wet weight) was taken for the isolation of nuclei.

The aliquot of homogenate, about 40 ml, was poured through 12 layers of cheesecloth and layered over 3 ml of Solution A in a Beckman cellulose nitrate tube (1 x 3.5 inches). The tubes were centrifuged at 70,000 x g (average) for 1 hour in a Beckman L2-65B Ultracentrifuge equipped with a SW 27 rotor. The nuclear pellet was resuspended with a loose fitting teflon homogenizer in 5 ml of 1.7 M sucrose in TKM buffer and filtered through organza cloth. The filtrate was layered over 15 ml of 1.8 M sucrose in TKM buffer in a 38 ml tube and centrifuged for 20 minutes at 15,000 x g in a Sorvall RC2-B centrifuge (Newtown, Conn.) equipped with a SS 34 rotor. The nuclear pellet was resuspended in 1 ml of 25% glycerol in 0.01 M Tris-HCl and 1 mM MgCl$_2$ adjusted to pH 7.9.

The purity of nuclei prepared by Method I was determined by three methods. First, an aliquot of the glycerol solution of nuclei was placed on a microscope slide and a drop of a 1% solution of toluidine blue in the same solution was added. The slide was examined by light microscopy
for evidence of broken nuclei, free nucleoli, or cellular debris. Each nuclear preparation was examined by light microscopy and the concentration of nuclei was determined by hemacytometer counting.

Six nuclear preparations by Method I were analyzed for DNA, RNA and protein to compare the results obtained by this method to those of published values, Table 1. The percent recovery was determined from the amount of DNA in the aliquot of homogenate, assuming that 100% of the DNA was present in the nucleus. The protein to DNA ratio was slightly high, and, therefore, it was suspected that cytoplasmic "tags" had adhered to the nuclear envelope during centrifugation through 2.0 M sucrose. However, electron microscopy, described in Appendix I, of a typical nuclear preparation indicated that nuclear morphology was similar to that published by Spelsberg et al (1974), Figure 1.

Preparation of Rat Liver Nuclei-Method II

Nuclei prepared by Method II were used to study the uptake of radioactivity from $^{14}$C-phenobarbital. Method II is essentially the method of Sadowski and Steiner (1968), chosen because the nuclei retain biochemical as well as morphological integrity. These characteristics were documented with light and electron micrographs, enzymatic studies with marker enzymes, and the chemical composition of both nuclei and nucleoli.
TABLE 1

NUCLEIC ACID AND PROTEIN CONTENT OF NUCLEI ISOLATED FROM THE LIVERS OF UNTREATED RATS\textsuperscript{a}

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<tr>
<td>RNA/DNA 0.3-0.4</td>
<td>Protein/DNA 2.5-3.0</td>
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<td>0.108</td>
<td>1.5</td>
<td>65.5</td>
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<tr>
<td>0.34</td>
<td>3.2</td>
<td>50.0</td>
</tr>
<tr>
<td>0.27</td>
<td>2.5</td>
<td>49.5</td>
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\textsuperscript{a} Livers from untreated rats were used for the isolation of nuclei as described in Methods. Values are the means of the ratios calculated for 6 rat livers.

\textsuperscript{b} The % recovery of nuclei was determined from the % recovery of DNA measured in liver homogenates and isolated nuclei.
FIGURE 1

Rat Liver Nucleus

Nuclei were prepared according to Method I as described in Methods and prepared for electron microscopy as described in the Appendix. Magnification is x 24,700.
Rats were killed by a rapid blow to the head, and livers were perfused in situ via the portal vein with cold 0.25 M sucrose containing 5 mM MgSO$_4$ (Medium H). All subsequent operations were conducted at 4°. The livers were rinsed, blotted, weighed and passed through a Harvard tissue press (Harvard Apparatus Sales Corp., Millis, Mass.) into a Potter-Elvehjem type homogenizer. Four volumes of Medium H were added, and the livers homogenized with six strokes. The homogenate was filtered through 12 layers of cheesecloth and centrifuged at 750 x g for 10 minutes in a Sorvall RC2-B centrifuge equipped with a SS 34 rotor. The pellet was resuspended in 3 volumes of Medium H containing 0.5% Triton X-100, layered over 15 ml 0.88 M sucrose containing 1.5 mM CaCl$_2$, and centrifuged for 10 minutes at 4,000 x g using the same rotor. The pellet was resuspended in 2.2 M sucrose containing 5 mM CaCl$_2$ and centrifuged for 1 hour at 100,000 x g in the Beckman ultracentrifuge using a SW 27 rotor. The nuclear pellet was resuspended in 1 volume of Medium H and was used immediately or stored at 0° for not more than 8 hours.

The results of assays for nucleic acids and protein of these nuclei, Table 1, indicated that RNA as well as protein were somewhat higher than that reported by Sadowski and Steiner (1968), Table 1, and the percent recovery was lower. However, light microscopy indicated that gross contamination of the nuclei had not occurred, and the isolated nuclei were intact.
Assay for Endogeneous DNA-Dependent RNA Polymerase Activity

Nuclei were isolated by Method I, described above, and used in the assay the same day. An aliquot of the isolated nuclei to be used was examined by light microscopy to insure no gross contamination by cellular debris and that the nuclei were intact with normal gross morphology.

The reaction to assay for DNA-dependent RNA polymerase activity was conducted in a room with the temperature controlled at 15°. A Dubnoff metabolic shaker and all the reagents to be used as well as the nuclear preparation were equilibrated to 15° for 1 hour prior to the assay. At 15° the activity of ribonuclease is nil, thus assuring that changes in the reaction product were not due to an effect of the drug on this enzyme.

The assay conditions were essentially according to Glasser and Spelsberg, 1972. Each assay contained in a total volume of 250 µl: 50 µl of the nuclear preparation; 10 µmoles of Tris-HCl buffer with the pH adjusted to 7.9; 0.16 µmole each of GTP, CTP and ATP (Sigma Chemical Company); 0.05 µmole of ³H-UTP (New England Nuclear) providing 2.5 µCi per assay; 0.1 µmole KH₂PO₄-NaOH buffer with the pH adjusted to 7.5; and 2 µmoles of KCl.

To the reactions designed to assay for RNA polymerase I, 0.5 µmole MgCl₂ was added, while to the reactions designed to assay for RNA polymerase II, 0.4 µmole MnCl₂ and 0.25 M (NH₄)₂SO₄ were added. The reaction was initiated
by the addition of nuclei and was terminated after 10 minutes by adding 1 ml of ice cold 10% (w/v) trichloroacetic acid (TCA).

The white, acid insoluble material was easily separated from the clear liquid by centrifugation at 2,000 rpm for 10 minutes in a Sorvall GLC-1 centrifuge, equipped with a type HL-4 rotor, located in a room with the temperature maintained at 4°. Centrifugation for less than 10 minutes at 2,000 rpm produced a pellet from which the supernatant could not be decanted without dislodging some of the material from the pellet. The pellet was washed twice with 2 ml of ice cold 5% (w/v) TCA containing 1% Na₂P₂O₇ (a polymerase inhibitor). After each wash the acid insoluble material was separated from the aqueous wash by centrifugation. It was essential that each resuspension of the pellet in the wash medium was performed carefully, since all of the unincorporated ³H-UTP must be removed from the acid insoluble material.

The pellet from the second wash was resuspended in the same medium and poured into the chimney of a Millipore filter (0.45 µ pore size). The Millipore filters were connected to a Plexiglas manifold which was maintained under vacuum. The material maintained on the filter was washed once with the cold TCA-Na₂P₂O₇ medium, and once with 2 ml of toluene to remove the TCA. The filters were dried for at least 30 minutes at room temperature. The dried filters were placed horizontally in the bottom of
25 mm diameter glass scintillation counting vials, and 10 ml of a cocktail composed of toluene containing 0.4% PPO and 0.01% POPOP. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instruments, Downers Grove, Illinois).

Aqueous standards of $^3$H-UTP containing 100 pmoles were applied to filters, dried and added to scintillation vials as described above. The 100 pmoles standards, corrected for background, counted at 14% efficiency and 1,500 counts per minute (cpm). Samples were analyzed for radioactivity under conditions identical to the standards, and cpm converted to disintegrations per minute (dpm) using the counting efficiency determined for the standards. The cpm determined from automatic external standards indicated that the counting efficiency did not change from sample to sample.

Initial results from experiments measuring the incorporation of radioactivity from $^3$H-UTP into acid insoluble material (RNA) during incubations of isolated nuclei were not reproducible. The standard deviation of triplicate determinations was often greater than 50% of the mean. The most likely explanation for excessive variation in replicate determinations was loss of material during extensive washing of the acid precipitate. Therefore, to more accurately estimate the recovery of the RNA on the filters, the amount of DNA which had co-precipitated was measured. This technique, apparently first reported by
Glasser and Spelsberg (1972), reduced the standard deviation of triplicate determinations from greater than 50% to less than 15% of the mean. Briefly, the method is as follows.

The filters, which had previously been assayed for radioactivity, were removed from the scintillation vials, dried and placed in test tubes with 1 ml of 0.3 M HClO₄ at 90° for 30 minutes to solubilize the DNA. The hydrolysate was used to determine the amount of DNA on each filter by the diphenylamine method of Burton (1956), as modified by Giles and Myers (1965). The dpm of ^3H-UTP and mg DNA on each filter were used to calculate the incorporation of UMP into RNA as pmoles UMP per mg DNA.

Measurement of Polyamines

Rats were injected intraperitoneally with 80 mg sodium phenobarbital/kg in saline or an equal volume of saline and killed at selected times in groups of three. Livers were quickly removed and extracted according to the method of Raina (1964).

Briefly, the livers were homogenized in 0.1 N HCl, deproteinized by the addition of 10% TCA, filtered and the TCA extracted from the filtrate with ethyl ether. The TCA free filtrate was made basic with NaOH and the amines extracted into n-butanol. The butanol layer was acidified and evaporated to dryness in a Buchler Rotary Evaporator (Buchler Instruments, Fort Lee, New Jersey). The residue
containing the polyamines, was dissolved in 0.5 ml of 0.1 N HCl, and a 20 μl aliquot was applied to a center line on Whatman No. 1 paper, 46 x 57 cm.

The polyamines were separated by high voltage electrophoresis in a Plexiglas tank, Model ET 48, and a high voltage power supply, Model EHV 2000, obtained from Servonuclear Corporation, Long Island City, New York.

Electrophoresis of samples was conducted at 400 volts for 90 minutes using 0.065 M sulphosalicylic acid adjusted to pH 3.5 with NaOH. Under these conditions the dissipation of heat was sufficient to allow operation at room temperature.

Following electrophoresis the paper was removed and dried at room temperature. The polyamines were stained by drawing the paper through a trough containing a saturated solution of amido black in methanol:acetic acid:water (2:1:7), and the paper dried in an oven at 100° for 5 minutes. The excess dye was removed by a series of washes in methanol-acetic acid (9:1).

The polyamines form complexes with amido black which are insoluble in the alcoholic wash and remain impregnated in the paper. The paper, with blue-black spots now clearly visible, was again dried, and the polyamine spots and blank areas of equal size were cut from the paper. The strips were eluted into 3 ml cuvettes with 0.1 N NaOH, and the absorbance at 615 nm measured with a Gilford Model 240 spectrophotometer (Gilford Instruments, Yellow Springs, OH).
The extraction recoveries of the polyamines were determined by adding known amounts of liver homogenates, extracting by the procedure described above, and determining the absorbance as a function of the amount added to the homogenate compared to the absorbance of standards added directly to 0.1 N NaOH. The recoveries of spermine or spermidine were greater than 90%, whereas the recovery of putrescine was poor and not reproducible. The standard curves for spermine and spermidine, corrected for their recoveries, were linear to at least 50 μmoles, and 5 μm moles could be detected reproducibly.

Since apparently the putrescine-amido black complex was too soluble in methanol-acetic acid to be quantified, putrescine was determined by a ninhydrin procedure reported by Janne (1967). The butanol extraction procedure, described above, was used, but electrophoresis was conducted in 0.1 M citric acid buffer adjusted to pH 4.3 for 60 minutes at 400 volts and 35 milliamps. Under these conditions the polyamines migrated the following distances: putrescine, 53 mm; spermidine, 40 mm; and spermine, 32 mm.

Following electrophoresis the paper was dried and sprayed with a solution composed of 1 g of ninhydrin in acetone:acetic acid:water (100:5:10) containing 1 mg/ml of cadmium acetate (Heilmann, 1957). Although not reported by Heilmann, it was found that the reagents for the spray
solution must be added in the sequence described above, lest apparently insoluble precipitates of cadmium form.

The sprayed paper was dried in an oven at 75° for 90 minutes. In order to maintain minimal background levels on the paper, a watch glass containing concentrated H₂SO₄ was put in the oven. After drying the colored spots, along with blanks of equal area, were cut and eluted with a solution of water:acetic acid:ethanol (1:5:4) containing 2 mg/ml of cadmium acetate. The absorption at 505 nm of the eluates was determined within 1 hour in cuvettes with a 1 cm light path in a Gilford spectrophotometer. Absorption was found to be linear to at least 0.650, and 30 µ moles gave the following absorptions: putrescine, 0.248; spermidine, 0.350; and sperimine, 0.463.

**Assay of Ornithine Decarboxylase Activity**

The rate limiting step for the synthesis of polyamines in the rat liver is the decarboxylation of ornithine to putrescine, catalyzed by the soluble enzyme ornithine decarboxylase (Tabor and Tabor, 1964). In order to examine the effect of in vivo phenobarbital treatment on the activity of this enzyme, an in vitro assay for ornithine decarboxylase reported by Russel and Snyder (1968) was modified and used as follows.

Groups of three rats were injected with either 80 mg sodium phenobarbital/kg in saline or an equal volume of saline and killed at selected times by a blow to the head.
Livers were quickly removed and homogenized in 5 volumes of cold 0.05 M sodium-potassium phosphate buffer adjusted to pH 7.2. The buffer contained 1mM 2-mercaptoethanol to stabilize the enzyme (Pegg and Williams-Ashman, 1968). The homogenate was centrifuged at 20,000 x g for 20 minutes in a Sorvall RC2-B centrifuge with a SS 34 rotor. The supernatant fluid was used for the assay of ornithine decarboxylase activity.

The reaction was conducted in 10 ml Erlenmeyer flasks equipped with rubber stoppers and polyethylene center wells obtained from Kontes Glass Company, Vineland, New Jersey. The center well contained 0.2 ml of a 2:1 solution of ethanol-amine and 2-methoxyethanol (Eastman Organic Chemicals, Rochester, New York). The reaction mixture contained:

- 0.1 μmole pyridoxal phosphate (Sigma),
- 2.5 μmole dithiothreitol (Calbiochem),
- 0.5 μCi D,L-ornithine-1-14C (New England, Nuclear),
- 2.0 μmoles L-ornithine (Sigma),
- 0.5 ml liver enzyme preparation

made up to a total volume of 2.0 ml with 0.05 M sodium-potassium phosphate buffer adjusted to pH 7.2.

The reaction was initiated by the addition of the substrate after a 5 minute pre-incubation of the reaction mixture at 37° in a Dubnoff metabolic incubator. The agitation was controlled at 120 cycles per minute. After 6 minutes incubation at 37° the reaction was terminated by the injection of 0.5 ml of 5 N H2SO4 through the rubber
stopper. The flasks were allowed to stand at room temperature for 30 minutes to allow the completion of the reaction of $^{14}\text{CO}_2$ with ethanolamine to form the carbonate in the center well.

The center well and its contents were placed in scintillation vials which contained 2 ml of ethanol and 10 ml of a cocktail composed of toluene, PPO and POPOP described previously. The radioactivity in the vials was determined in a Packard Tri-Carb liquid scintillation spectrometer at 40% counting efficiency. Radioactivity was corrected for the nonenzymatic release of $^{14}\text{CO}_2$ from the reaction by subtracting radioactivity released from triplicate reactions containing a previously boiled enzyme preparation. The data were expressed as mmol of $^{14}\text{CO}_2$ released per hour per gram of liver (wet weight). Although the substrate contained $^{14}\text{C-D-ornithine}$ as well as $^{14}\text{C-L-ornithine}$, it was assumed that only the L-form of the substrate was enzymatically decarboxylated. Therefore, the calculations were based only on the specific activity of L-ornithine-1-$^{14}\text{C}$.

**Fractionation of Rat Liver to Determine the Subcellular Distribution of Phenobarbital**

The subcellular distribution of phenobarbital in rat liver as a function of time was estimated by measurement of radioactivity from $^3\text{H}$-phenobarbital in isolated subcellular fractions. Rats weighing 140 to 160 g were injected intraperitoneally with 80 mg $^3\text{H}$-phenobarbital/kg, specific
activity 0.7 mCi/m mole, and killed in groups of three at selected times by decapitation. Blood was collected from the cervical region and allowed to clot. The liver was quickly removed and divided into two 2 g aliquots.

One aliquot was used to prepare nuclei according to Method I described above. The other aliquot of liver was homogenized in 4 volumes of TKM buffer and centrifuged at 9,000 x g for 15 minutes in a Sorvall RC2-B centrifuge using a SS 34 rotor. The 9,000 x g supernatant fraction was then centrifuged at 100,000 x g for 60 minutes in a Beckman L2-65B centrifuge using a SW 27 rotor. The pellicle and floating lipid layer were discarded and the supernatant fluid decanted and saved. The pellet was resuspended in 1.15% KCl and recentrifuged at 100,000 x g for 60 minutes. In a preliminary experiment it had been determined that a single KCl wash was sufficient to remove radioactivity which was loosely bound.

The KCl washed pellet was designated the microsomal fraction. The supernatant fluid obtained from centrifugation at 100,000 x g was designated the cytosol, i.e., the non-particulate fraction derived from the cytoplasm. Aliquots of the microsomal fraction, resuspended in 1.15% KCl, nuclei, serum, liver homogenate or the cytosol were placed in scintillation vials and 10 ml of Unogel (Schwarz/ Mann, Orangeburg, New Jersey) were added. Radioactivity was determined in a Packard Tri-Carb liquid scintillation
spectrometer. The counting efficiency of each sample was determined by counting an internal standard, and was about 20%.

Aliquots of the microsomal fraction, the cytosol, and the nuclear fraction were analyzed for protein by the method of Lowry et al (1951) using bovine serum albumin as the standard.

The radioactivity determined for each aliquot was expressed as µg equivalents (µg eq) of phenobarbital since it was not known whether the radioactivity was associated with phenobarbital per se or with metabolites of phenobarbital. Radioactivity was normalized either to the protein content of the aliquot, µg eq/mg of protein, or to the original wet weight of the liver, µg eq/g of liver.

Isolation and Measurement of RNA, DNA and Protein From Rat Liver Nuclei

In order to characterize the nuclear preparations of Method I and Method II described above, the concentrations of nucleic acids and protein were determined on nuclei isolated by each method from two groups of six untreated rats. Nuclear pellets were resuspended in 0.25 M sucrose and fractionated according to Method II of Shibko et al (1967). RNA was determined by the orcinol reaction described by Drury (1948) as modified by Schneider (1957). DNA was determined by the Giles and Myers (1965) modification of the diphenylamine reaction of Burton (1956),
using calf thymus DNA as the standard. Diphenylamine was purified by precipitation from a saturated methanol solution by the addition of water. Even recently purchased diphenylamine had to be purified in order to avoid cloudy suspensions. Protein was determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Measurement of Drug Binding to Protein in the Nucleoplasm or the Cytosol

Drug-protein binding was determined by gel filtration chromatography of aliquots of nucleoplasm or cytosol prepared from the livers of rats which had been given tracer doses of $^{3}H$-phenobarbital.

Rats were injected intraperitoneally with 50 μCi of phenobarbital, specific activity 10.3 Ci/mmole, in saline solution and killed at either 5 or 20 minutes by a blow to the head. All subsequent steps were conducted at 4° unless otherwise indicated. Livers were perfused \textit{in situ} via the hepatic portal vein with about 15 ml of a solution composed of 0.25 M sucrose containing 5 mM MgSO$_4$ (Medium H). The livers were homogenized in 4 volumes of Medium H with six strokes of a Potter-Elvehjem type homogenizer equipped with a teflon pestle.

One aliquot of the homogenate was diluted with Medium H and nuclei were isolated according to Method II. The remaining homogenate was used to prepare the cytosol as described above.

Sephadex G-25 (fine) and Sephadex G-50 were obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. The
gels were prepared by swelling in a buffer composed of 0.05 M tris-HCl containing 0.05 M KCl adjusted to pH 8.0. The volume of buffer was a 5-fold excess of the column capacity. Swelling time was shortened and air bubbles reduced by placing the beakers in a 90° water bath for 1 or 5 hours for the G-25 or G-50 gels, respectively. The slurries were poured into glass columns, 1.5 x 43 or 1.5 x 25 cm for the G-25 or G-50 gels, respectively.

A reservoir containing Medium H was connected by siphon to the column. The column effluent was attached to a flow-through cell with a 25 μl capacity and 0.25 cm light path (Gilford Instruments, Yellow Springs, Ohio), and the absorbance at 280 nm was monitored continuously by a Gilford model 240 spectrophotometer and recorded by a Heath Servorecorder, Model EU-20B (Heath Company, Benton Harbor, Michigan). The effluent from the flow-through cell was collected in an Isco fraction collector, Model 321 (Instrumentation Specialties Company, Inc., Lincoln, Nebraska). Approximately 4 ml fractions were collected, and 1 ml aliquots were analyzed for radioactivity by liquid scintillation spectrometry. Internal standards were used to correct for quenching.

Nucleoplasm was prepared by the disruption of nuclei, prepared by Method II, by sonication with a Biosonik IV (Bronwill Corporation, Rochester, New York) at the maximum setting for 15 seconds. The disrupted nuclei were centrifuged at 2,000 rpm in a Sorvall GLC-1 centrifuge, and the supernatant fluid was designated the nucleoplasm.
Aliquots of nucleoplasm or cytosol were analyzed for radioactivity to determine the total radioactivity applied to each column. Approximately 3 ml aliquots of nucleoplasm or cytosol were chromatographed.

**Measurement of ^14_C-Phenobarbital Uptake by Isolated Nuclei**

The *in vitro* uptake of ^14_C-phenobarbital into rat liver nuclei was measured by incubation of nuclei and ^14_C-phenobarbital in a physiological medium containing various amounts of cytosol, reisolating the nuclei under conditions which would remove drug loosely attached to the exterior of the nuclei, and determining the amount of radioactivity associated with the reisolated nuclei.

Nuclei were isolated by Method II above, and cytosol was prepared as described. The reaction mixture contained in a total volume of 3 ml:

- 4 mM MgCl$_2$
- 16 mM KCl
- 2 mM ATP
- 8 mM 2-mercaptoethanol
- 5 mM D-glucose
- 90 μM ^14_C-phenobarbital
- 0.1 M tris-HCl buffer (pH 7.4)

and various amounts of nuclei and cytosol. The reaction was conducted in a Dubnoff metabolic shaking incubator at 120 cycles per minute and under oxygen:CO$_2$ (95:5) at 37° for 1 hour. Controls were incubated at 4° for 1 hour. The reactions were terminated by plunging the flasks into ice.
The reaction mixture and a 1 ml rinse composed of 0.1 M tris-HCl buffer adjusted to pH 7.4 were layered over 15 ml of 1.7 M sucrose containing 3 mM CaCl₂ in a 50 ml cellulose nitrate centrifuge tube. Intact nuclei were centrifuged through the sucrose at 70,000 x g for 30 minutes in a Beckman ultracentrifuge using a SW 27 rotor.

The supernatant fluid was decanted, the tube rinsed and wiped dry and the pellet of nuclei was suspended in 1 ml water. The suspension was drawn into a 1 ml pipette, the volume measured, and dispensed into a scintillation vial. Radioactivity was analyzed in a 10 ml Unogel cocktail by liquid scintillation spectrometry at 70% efficiency determined by counting internal standards.
RESULTS

The Distribution of $^{3}$H-Phenobarbital in Rat Liver and Serum After Intraperitoneal Administration

Although phenobarbital causes gross alterations in rat liver size and metabolic activity only after repeated high doses of the drug, more recent investigations on rat liver biochemistry suggest that changes which precede detectable induction may occur within hours of drug administration. In order to estimate the time when the earliest alterations in rat liver biochemistry may occur, it was postulated that entry of the drug into the cell cytosol or nucleus may be rate limiting. Therefore, radioactivity from radiolabeled phenobarbital administered at a dose of 80 mg/kg intraperitoneally was measured as a function of time in rat liver cytosol, nuclei, microsomes and serum, Figures 2 and 3.

The concentration of radioactivity, expressed as µg equivalents phenobarbital/g of liver (µg eq/g) was initially higher in liver cytosol than in the serum, Figure 2. The concentration of the drug in liver cytosol of rats killed at 1 minute was about 75% of the maximum concentration attained at 10 minutes. The drug concentration in the serum was not statistically different from the
Rats were injected with 80 mg $^3$H-phenobarbital/kg ip (specific activity, 3.0 μCi/mg) and killed at the indicated times. Subcellular fractions were prepared as described in Methods. Serum was obtained from blood collected at the time of sacrifice and the concentration is expressed as μg eq of phenobarbital/ml of serum. Points with vertical bars represent the mean and standard error of 3 rats. Points without vertical bars are single determinations.
FIGURE 2

μg eq Phenobarbital/gram Liver or Serum, Mean ± SEM

Minutes After Phenobarbital Treatment
FIGURE 3

Distribution of Phenobarbital in Rat Liver
Microsomes and Nuclei

Rats were injected with 80 mg $^3$H-phenobarbital/kg ip (specific activity, 3.0 μCi/mg) and killed at the indicated times. Microsomes and nuclei were prepared as described in Methods. The protein content of each fraction was determined according to the method of Lowry, et al., (1951). Each point is the average of at least 3 separate animals and vertical bars represent standard errors of the means.
FIGURE 3

Minutes After Phenobarbital Treatment

Ng eq Phenobarbital/mg Microsomal Protein, Mean ± SEM

Ng eq Phenobarbital/mg Nuclear Protein, Mean ± SEM

Microsomes

Nuclei
concentration in the cytosol from 10 to 120 minutes. Measurements of radioactivity in liver homogenates indicated that about 20% of the radioactivity was not accounted for in the cytosol and microsomal fractions, but probably was located in the lipid and mitochondrial fractions, which were not analyzed.

Since most of the studies reported herein were conducted within 1 hour of drug administration, it should be emphasized that the drug concentration in liver cytosol between 1 and 60 minutes increased from 132 to 178 µg eq/g of liver. After 10 minutes the concentration declined with a half life value of 9.9 ± 0.6 hours, mean and 95% confidence limits. The half life value was determined by linear regression analysis of the logarithmically transformed data. The volume of distribution of the drug was 48% of the body weight, determined by extrapolation of the serum concentration curve to zero time (Notari, 1971).

Measurement of radioactivity in nuclei and the microsomal fraction of rat liver from 1 to 120 minutes indicated that the drug and/or metabolites accumulated as a function of time in the microsomal fraction but not in nuclei. Since the microsomal fraction was washed as described in Methods, radioactivity associated with this fraction was bound by forces stronger than those disrupted by the KCl solution. The accumulation of the drug by the microsomal fraction occurred in spite of a small decrease in its concentration in the cytosol, Figure 2.
In contrast to the microsomal fraction, nuclei did not apparently accumulate the drug between 10 and 120 minutes, Figure 3. However, nuclei isolated 1 minute after drug administration contained more radioactivity than at any other time examined. The standard error of the mean for the concentration of radioactivity in nuclei at 1 minute was larger than for radioactivity in any other subcellular fraction measured, but the concentrations at 30, 60 and 120 minutes were statistically lower than at 1 minute.

Based on the distribution of radioactivity it was clear that phenobarbital was at least available in the liver cytoplasm and nuclei as early as 1 minute after an intraperitoneal injection. Therefore, if phenobarbital were to have a direct effect on the synthesis of new enzyme protein, this effect might occur within minutes after administration of the drug.

The Activity of DNA-Dependent RNA Polymerase (I and II) After Phenobarbital Administration in Vivo

In subsequent experiments the activities of DNA-dependent RNA polymerase I and II were measured as a function of time after administration of 80 mg phenobarbital/kg. In Figure 4 are shown the activities of polymerase I or II expressed as the percentages of the respective polymerase activities in control animals. Control rats were injected with an equal volume of saline, and
Activities of Rat Liver RNA Polymerase I and II After Phenobarbital Treatment

Rats were treated at approximately 10:00 a.m. with 80 mg phenobarbital/kg ip and killed at the indicated times. Nuclei were prepared from livers as described in Methods. Each point on the graph represents the average from at least two animals, and each assay was run in duplicate. Vertical bars represent the standard error of the mean. The activity for polymerase I in livers from control rats was 27 pmoles $^{3}$H-UMP/mg DNA, and for polymerase II was 140 pmoles $^{3}$H-UMP/mg DNA.

Each assay contained in a total volume of 250 ul:
50 µl nuclei (approximately 100 µg DNA), 10 µmoles Tris-HCl (pH 7.5), 0.16 µmole each of GTP, CTP, and ATP, 0.05 µmole $^{3}$H-UTP (2.5 µCi), 0.1 µmole phosphate buffer (pH 7.5), and 2 µmoles KCl. Assays for polymerase I contained 0.5 µmole MgCl$_2$, and assays for polymerase II contained 0.4 µmole MnCl$_2$ and 0.25 M (NH$_4$)$_2$SO$_4$. Reactions were run for 10 minutes at 15°C. DNA was measured for each assay by the diphenylamine reaction (see Methods).
FIGURE 4

% of Control, Mean ± SEM

- O Polymerase I (Nucleolar)
- O Polymerase II (Nuclear)

Minutes After Phenobarbital Treatment
the polymerase activities did not change significantly between zero and 60 minutes. Therefore, the results of all control animals were pooled. The values of RNA polymerase I and II activity for control rats were 27 and 140 p moles $^3$H-UMP incorporated per mg of DNA, respectively.

The activity of RNA polymerase II, which synthesizes messenger-like RNA, did not change significantly at 5, 10, 20, 30 or 60 minutes after phenobarbital treatment. However, the activity of RNA polymerase I, which synthesizes ribosomal-like RNA, was significantly elevated at 5, 10 and 30 minutes to 220, 165 and 180% of control activity, respectively. The activity of RNA polymerase I at 20 and 60 minutes was not significantly different from controls at the 0.05 level of significance using Student's t test.

Thus, the stimulation of RNA polymerase I activity was greatest, 220% of control, at the earliest time measured, 5 minutes. The second peak of activity, at 30 minutes occurred after a return to near control levels at 20 minutes, and thereby appeared to be a separate event. It was postulated that the initial stimulation of RNA polymerase I activity might be due to a direct effect of the drug on the enzyme, since polymerase I was apparently stimulated as soon as the drug entered the liver cell, Figures 2 and 3.
The Activity of DNA-Dependent RNA Polymerase I: Effect of Phenobarbital or Cytosol Added in Vitro

In an attempt to demonstrate a direct effect of phenobarbital on the activity of RNA polymerase I in vitro, rats were killed after no drug treatment, and nuclei were prepared and isolated as described in Methods. Activities of either RNA polymerase I or II were measured in the same assay described in Methods for nuclei isolated from treated rats, except that from $10^{-7}$ to $10^{-5}$ M sodium phenobarbital in solution in the tris buffer was added to the reaction mixture. Cytosol was prepared from the livers of rats either untreated or treated with 80 mg phenobarbital/kg intraperitoneally and killed 5 or 30 minutes later. Cytosol was prepared from a 25% (w/v) homogenate of liver in 0.05 M Tris HCl buffer, adjusted to pH 7.9, by centrifugation at 100,000 xg for 60 minutes. Cytosol, either 10 or 100 mg, was added to selected assays for RNA polymerase I or II, Tables 2 and 3.

In Table 2 is shown the effect of added phenobarbital and/or cytosol from livers of phenobarbital treated or untreated rats on the activity of RNA polymerase I. The activity of RNA polymerase I was stimulated to 300% of control activity by the addition of 100 mg of cytosol from untreated rats, Table 2A. No further stimulation was obtained by adding from $10^{-7}$ to $10^{-5}$ M phenobarbital to the reaction mixture.
TABLE 2

EFFECT OF PHENOBARBITAL OR LIVER CYTOSOL ON THE ACTIVITY OF RNA POLYMERASE I IN VITRO

<table>
<thead>
<tr>
<th>Phenobarbital(^b, M)</th>
<th>Cytosol(^c)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Time, Min.</td>
</tr>
<tr>
<td>A.</td>
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<tr>
<td>(10^{-7})</td>
<td>0</td>
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<tr>
<td>(10^{-6})</td>
<td>0</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td>0</td>
</tr>
<tr>
<td>B.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td>5</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td>30</td>
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<tr>
<td>C.</td>
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</tr>
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<td>(10^{-5})</td>
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<tr>
<td>(10^{-5})</td>
<td>5</td>
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<tr>
<td>(10^{-5})</td>
<td>30</td>
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<tr>
<td>D.</td>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td>5</td>
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<tr>
<td>(10^{-5})</td>
<td>30</td>
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<td>E.</td>
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<td>(10^{-5})</td>
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<td>(10^{-5})</td>
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</table>

a. Nuclei were prepared from livers of untreated rats by Method I as described in Methods. The activity of RNA polymerase I was determined in vitro as described in Methods. Duplicate assays were run and values were averaged.

b. Phenobarbital was added to the reaction mixture in Tris-HCl buffer.

c. Cytosol was prepared from livers of rats either untreated (0 time) or pretreated with 80 mg phenobarbital/kg for 5 or 30 minutes. Either 10 or 100 mg of cytosol was added to the reaction mixture in Tris-HCl buffer. The volume of the reaction mixture was kept constant.
TABLE 3

EFFECT OF PHENOBARBITAL OR LIVER CYTOSOL ON THE
ACTIVITY OF RNA POLYMERASE II IN VITRO\textsuperscript{a}

<table>
<thead>
<tr>
<th>Phenobarbital\textsuperscript{b}, M</th>
<th>Cytosol\textsuperscript{c}</th>
<th>Time, Min.</th>
<th>mg</th>
<th>% Control</th>
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<tr>
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<tr>
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<tr>
<td>(10^{-5})</td>
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<td>103</td>
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<td>30</td>
<td>100</td>
<td>82</td>
</tr>
<tr>
<td>C. (10^{-7})</td>
<td></td>
<td>0</td>
<td>100</td>
<td>85</td>
</tr>
<tr>
<td>(10^{-6})</td>
<td></td>
<td>0</td>
<td>100</td>
<td>104</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td></td>
<td>0</td>
<td>100</td>
<td>102</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Nuclei were prepared from livers of untreated rats by Method I as described in Methods. The activity of RNA polymerase II was determined in vitro as described in Methods. Duplicate assays were run, and values are averages.

\textsuperscript{b} Phenobarbital was added to the reaction mixture in Tris-HCl buffer.

\textsuperscript{c} Cytosol was prepared from livers of rats either untreated (0 time) or pretreated with 80 mg phenobarbital/kg ip for 5 or 30 minutes. Either 10 or 100 mg of cytosol was added to the reaction mixture in Tris-HCl buffer. The volume of the reaction mixture was kept constant.
Since cytosol added in vitro stimulated RNA polymerase I activity, it was postulated that a factor may be present in liver cytosol which is required in the presence of phenobarbital to activate RNA polymerase I. It was further postulated that this factor may be depleted or reduced by phenobarbital treatment. Therefore, 100 mg of cytosol from rats either untreated (0 minutes) or treated for 5 or 30 minutes were added to the reaction mixture, Table 2B. There was a trend for the activity of RNA polymerase I to decrease with added cytosol from rats pretreated for 5 and 30 minutes, from 300% of control to 262 and 252%. The same experiment was conducted in the presence of $10^{-5}$ M phenobarbital, Table 2C, with essentially the same results. The decrease in the stimulated activity of RNA polymerase I by cytosol from rats treated for 5 or 30 minutes was from 302% of control to 268% and 216%, respectively. Therefore, cytosol from rats pretreated with phenobarbital was less effective in stimulating RNA polymerase I activity than cytosol from untreated rats. The addition of phenobarbital in vitro either had no effect or was slightly inhibitory.

When 10 mg of cytosol from rats either untreated or treated with phenobarbital for 5 or 30 minutes was added to the reaction mixture in the absence of added phenobarbital, the stimulation of RNA polymerase I was about 25% of the stimulation produced by 100 mg of cytosol, Table 2D. The effect of phenobarbital pretreatment was not apparent when only 10 mg of cytosol was added.
The effect of added cytosol from untreated rats on the activity of RNA polymerase I in the presence or absence of added phenobarbital is shown in Table 2E and F, respectively. Phenobarbital added in vitro again had no apparent effect on the activity of RNA polymerase I, but the activity was stimulated slightly by 10 mg of cytosol, from 148 to 170% of control, whereas 100 mg of cytosol stimulated the activity to 300 and 302% of control. When 10 mg of cytosol from untreated rats was added to the reaction mixture in the presence of increasing amounts of phenobarbital, Table 2F, only the effect of added cytosol was apparent, a stimulation of the activity from 130 to 170% of control, and the added phenobarbital had no effect.

To summarize the findings presented in Table 2, the activity of RNA polymerase I in vitro was stimulated by the addition of cytosol. This stimulation was greater when 100 mg of cytosol was added than when 10 mg was added (about four fold). Cytosol from rats pretreated with phenobarbital was less stimulative than cytosol from untreated rats, and longer pretreatment with phenobarbital apparently resulted in cytosol which was less stimulatative than cytosol from rats pretreated for shorter periods of time, 0 or 5 minutes. Phenobarbital added in vitro apparently had no effect on the activity of RNA polymerase I or was slightly inhibitory.

These results are consistent with the hypothesis that a factor is present in rat liver cytoplasm which is capable
of stimulating the activity of RNA polymerase I, and that this factor is depleted by phenobarbital treatment. The depletion of a cytoplasmic factor is consistent with the postulate that this factor may enter the nucleus in the presence of phenobarbital to stimulate RNA polymerase I in vivo, but this factor may also be able to enter the nucleus under in vitro incubation conditions without the presence of phenobarbital.

The Activity of DNA-Dependent RNA Polymerase II: Effect of Phenobarbital or Cytosol Added in Vitro

The stimulation of RNA polymerase I activity in vitro by added cytosol was consistent with the stimulation produced by phenobarbital administration in vivo, Table 2 and Figure 4. However, no stimulation of the activity of RNA polymerase II was observed in rat liver nuclei after phenobarbital treatment, Figure 4. The effect of added phenobarbital or cytosol in vitro on the activity of RNA polymerase II was tested using methods described in the previous section and the assay for RNA polymerase II as described in Methods, Table 3.

When phenobarbital was added to the reaction mixture in concentrations from 0 to $10^{-5}$ M, the activity of RNA polymerase II was not changed from control activity, Table 2A. Added cytosol, 10 or 100 mg, from untreated rats had no effect or was slightly inhibitory, Table 3B. Added cytosol from rats pretreated with phenobarbital
for 5 or 30 mintues had no effect on RNA polymerase II activity, Table 3B. Added phenobarbital to reaction mixtures containing 100 mg of cytosol from untreated rats had no effect on the activity of RNA polymerase II activity, Table 3C.

Therefore, the lack of an effect of added cytosol or phenobarbital on the activity of RNA polymerase II, Table 3, is consistent with the lack of effect of phenobarbital treatment of rats on the activity of RNA polymerase II shown in Figure 4.

**Binding of $^{14}C$ or $^{3}H$-Phenobarbital to Proteins in Rat Liver Cytosol or Nucleoplasm**

To test the hypothesis that a factor located normally in the cytosol enters the nucleus in the presence of phenobarbital, the binding of $^{3}H$-phenobarbital to protein of rat liver cytosol or nucleoplasm was investigated using gel filtration chromatography. Since changes in the activity of RNA polymerase I had been observed in liver nuclei isolated from rats treated with phenobarbital for 5 minutes, Figure 4, the binding of radioactivity from $^{3}H$-phenobarbital to liver proteins was examined at 5 minutes. Binding was examined again at 20 minutes, when nuclear RNA polymerase I activity had returned to the control level of activity.
Initial experiments to demonstrate binding of radioactivity to proteins in liver cytosol from rats treated with 80 mg $^{14}$C-phenobarbital/kg did not reveal any detectable binding. It was postulated that the specific activity of the labeled drug was too low to measure small amounts of bound drug. Therefore, $^3$H-phenobarbital was administered to rats as a "tracer" dose, without dilution of the 10.3 Ci/mmole specific activity. Radioactive phenobarbital was administered intraperitoneally at a dose of 50 μCi to each rat. Rats were killed 5 or 20 minutes after treatment, and the cytosol and nucleoplasm were isolated as described in Methods.

In Figure 5 (top) is shown the column elution pattern of radioactivity, expressed as dpm, from the liver cytosol of rats treated with $^3$H-phenobarbital for 5 minutes. Radioactivity was plotted semilogarithmically to allow the display to the larger peaks on the same graph with the smaller peaks. At the bottom of Figure 5 is shown the column elution pattern of protein, expressed as the absorption measured at 280 nm, from the same fractions of rat liver cytosol as shown at the top. Radioactivity eluted from the column in five major peaks, identified in Figure 5 with Roman numerals. The majority of the radioactivity eluted in peak IV, and peaks II through V contained over 99% of the total radioactivity in the cytosol. Peak I eluted near the void volume of the column, 38 ml, indicating an association of the radio-
Rats were injected ip with 50 µCi $^3$H-phenobarbital (specific activity, 10.3 Ci/m mole) and killed 5 minutes later. Cytosol was prepared by homogenization of liver in sucrose-magnesium and centrifugation at 750 x g for 20 minutes and 100,000 x g for 60 minutes. Three ml of cytosol (equivalent to about 500 mg liver) was applied to a column 1.5 x 43 cm packed with Sephadex G-25. The cytosol applied to the column contained approximately $10^5$ dpm and counted at 20% efficiency. Fractions were 4 ml each, and the void volume of the column was 38 ml calibrated with Blue Dextran 2000 and bovine serum albumin. Fractions were monitored continuously at 280 nm.
FIGURE 5

[Graph showing dpm and absorbance at 280 nm against fraction number]
activity with proteins having a molecular weight of approximately 25,000 or greater. The total radioactivity associated with peak I represented only about 0.5% of the total radioactivity applied to the column. In subsequent column elutions of cytosol isolated from rats treated for 5 minutes, the radioactivity associated with peak I was always less than 1% of the total radioactivity applied to the column.

The location of unbound phenobarbital was determined by chromatography of the drug in TKM buffer in the absence of cytosol on the same column. The unbound drug eluted from the column in a volume approximately 3 times the void volume, and corresponded to peak IV in Figure 5. The majority of the protein, shown at the bottom of Figure 5, eluted with the radioactivity in peak I. A much smaller amount of protein eluted with the radioactivity in peak IV, corresponding to unbound drug. The protein which eluted with the radioactivity in peak V was found to have a low ratio of absorbance at 280 nm to absorbance at 260 nm, suggesting that the protein in peak V may have contained considerable amounts of RNA. Thus, the protein eluting with peak V may have been due to contamination of the cytosol with fragments of the microsomal fraction which did not sediment during the 100,000 x g centrifugation.

Fractions of column eluate composing each of the 5 peaks of radioactivity were combined and concentrated by ultrafiltration (Amicon, MMC) through a membrane which retains proteins having a nominal molecular weight greater
than 10,000 (Amicon, PM-10 membranes). The ultrafiltration was continued until the volume was reduced to 3 ml. Most of the radioactivity passed through the membrane during the ultrafiltration. The radioactivity which was retained with the protein was rechromatographed on the same Sephadex G-25 column. Rechromatography following ultrafiltration resulted in a shift of all detectable radioactivity to fractions 23 through 27, which corresponded to unbound drug. Other attempts to isolate and characterize the bound radioactivity, e.g., ammonium sulfate fractionation, resulted in loss of binding. The forces which retained radioactivity from \( ^3 \)H-phenobarbital in association with protein of rat liver cytosol during gel filtration chromatography were apparently very weak.

Cytosol from livers of rats treated with \( ^3 \)H-phenobarbital for 20 minutes was chromatographed on the same column used previously. The elution pattern of radioactivity and protein are shown in Figure 6 top and bottom, respectively. The elution of radioactivity and protein was similar in rats treated with \( ^3 \)H-phenobarbital for 5 or 20 minutes, but the striking difference was in the magnitude of peak I which had decreased from 1000 dpm, Figure 5, to less than 100 dpm at 20 minutes, Figure 6. The magnitude of peak I from liver cytosol of rats subsequently treated for 20 minutes was never found to be greater than about 100 dpm. The magnitude of other peaks of radioactivity (II through V) did not change in cytosol isolated from rats treated from 5 to 20
FIGURE 6

Binding of $^3$H-Phenobarbital to Rat Liver Cytosol at 20 minutes

Details of the procedures are given in the legend to Figure 5 and in Methods. Rats were killed 20 minutes after $^3$H-phenobarbital treatment.
minutes. Thus, between 5 and 20 minutes, the radioactivity associated with peak I had changed approximately 10 fold, while radioactivity associated with other peaks as well as the total radioactivity in the cytosol remained unchanged.

Based on the results of in vivo studies of phenobarbital binding to protein in liver cytosol, the question was raised whether phenobarbital would bind to protein eluting in peak I when added in vitro. Cytosol was prepared from a rat treated with $^3$H-phenobarbital for 20 minutes. Immediately prior to chromatography on a Sephadex G-25 column, approximately 1 μCi of $^{14}$C-phenobarbital (specific activity 3.1 mCi/m mole) was added to the cytosol, Figure 7. Radioactivity as well as absorbance at 280 nm was monitored as in previous experiments. Most of the $^{14}$C activity eluted with peak IV, and only small amounts were associated with peaks II and III. None of the $^{14}$C activity eluted with peak I, and apparently none eluted with peak V. Since $^3$H-phenobarbital in the absence of cytosol eluted in peak IV, it was concluded that the $^{14}$C activity eluting in peak IV was unbound drug. When the experiment was repeated using cytosol from untreated rats and either $^{14}$C or $^3$H-phenobarbital added in vitro, the majority of the radioactivity eluted in peak IV and none with peak I.

The apparent absence of binding of phenobarbital added to cytosol in vitro in the region of peak V (possibly microsomal fragments containing RNA) as well as the absence of in vitro binding to peak I suggested the possibility that
Rats were administered $^3$H-phenobarbital and liver cytosol was prepared as described in the legend to Figure 5. Immediately prior to chromatography on Sephadex G-25, $^{14}$C-phenobarbital (specific activity, 3.1 mCi/m mole) was added to the cytosol as a marker for zero time binding and to confirm the location of unbound drug eluting from the column. Liver cytosol was prepared from a rat treated for 20 minutes with $^3$H-phenobarbital.
FIGURE 7
radioactivity eluting in peak I was a biotransformation product of phenobarbital. In order to test this possibility, $^3$H-phenobarbital at concentrations of $10^{-7}$ or $10^{-5}$M was added to an incubation mixture composed of a 9,000 x g rat liver supernatant fraction and appropriate ions and co-factors required for in vitro drug metabolism (Bresnik, 1966). The reaction was conducted for 15 and 45 minutes and centrifuged at 100,000 x g for 60 minutes. The 100,000 x g supernatant fraction was chromatographed on a Sephadex G-50 column, and fractions collected and monitored for radioactivity and absorbance at 280 nm. The radioactivity associated with peak I was less than 100 dpm and did not increase when incubations were conducted from 15 to 45 minutes. Therefore, the association of radioactivity with peak I under in vivo conditions could not be duplicated in vitro, either by adding the drug directly to the cytosol, or by incubation in an in vitro drug metabolizing system.

The phenobarbital or metabolite which was associated with peak I at 5 minutes and decreased in magnitude by 20 minutes could have left the cytoplasm and entered the interstitial fluid, blood, bile, or a cellular organelle such as the nucleus. Since it was shown that RNA polymerase I activity in the nucleus had increased as early as 5 minutes after phenobarbital treatment, it seemed likely that radioactivity associated with peak I may have entered the nucleus.
Therefore, nuclei were prepared according to Method II described in Methods from rats treated with $^3$H-phenobarbital for 5 or 20 minutes. Nuclei were sonicated to disrupt the nuclear membranes, and nucleoplasm was prepared and chromatographed on a Sephadex G-25 column as described in Methods. Figure 8 shows the pattern of elution of radioactivity (top) and absorbance at 280 nm (bottom). Most of the $^3$H-phenobarbital or metabolites of phenobarbital found in the nucleoplasm isolated 5 minutes after treatment eluted in fractions 23 to 27, corresponding to unbound radioactivity. After 20 minutes of treatment with $^3$H-phenobarbital, radioactivity in the nucleoplasm eluted from the column in earlier fractions as well as in fractions corresponding to unbound radioactivity, indicating that at 20 minutes either phenobarbital or its metabolites were associated with proteins in the nucleoplasm. The pattern of absorbance at 280 nm, Figure 8 bottom, was the same for nucleoplasm isolated either at 5 or 20 minutes, suggesting that the association of radioactivity with protein was either to pre-existing material in the nucleoplasm or to new material which had entered the nucleoplasm, but in amounts too small to be detected. Rechromatography of nucleoplasm from rats treated for 20 minutes resulted in a shift in the radioactivity to fractions corresponding to unbound drug.

In the studies described above, an association of radioactivity from injected $^3$H-phenobarbital with protein
FIGURE 8

Binding of $^3$H-Phenobarbital to Rat Liver Nucleoplasm

Rats were injected ip with 50 µCi
$^3$H-phenobarbital (specific activity, 10.3 Ci/m mole)
and killed 5 or 20 minutes later. The livers were
perfused in situ, homogenized and nuclei prepared
according to Method II as described in Methods.
Nuclei equivalent to 3 grams of liver and about
4000 dpm were sonicated, centrifuged and applied to
a column of Sephadex G-25. Fractions of 4 ml were
monitored at 280nm and for radioactivity.
FIGURE 8

[Graph showing absorbance over time with different markers for 5 and 20 minutes.]
of rat liver cytosol as well as nucleoplasm was found, and this association was quantitatively dependent on time. This association with protein was apparently by forces much weaker than covalent bonding, and was disrupted by a variety of attempts to isolate or concentrate the complex. Since a time relationship existed between cytoplasmic and nucleoplasmic binding, it was postulated that the binding in the cytoplasm may be related to the uptake of the drug, or of the material to which the drug apparently binds, by nuclei. In order to test this proposition, the influence of cytosol on the uptake of the drug by isolated nuclei was evaluated.

**The Uptake of Phenobarbital by Isolated Nuclei**

Since the complex between $^3$H-phenobarbital and peak I protein of rat liver cytosol could not be isolated, the influence of the cytosol fraction of rat liver on the uptake of the drug by isolated rat liver nuclei was examined. Thus, if a protein in the liver cytosol associates with the drug to facilitate the uptake of the drug by nuclei, then the addition of cytosol to incubations of isolated nuclei should increase the uptake over nuclei incubated in the absence of cytosol.

Nuclei were isolated from the livers of untreated rats as described in Methods. Incubations were conducted at 37 or 4° in a physiological buffer solution containing 90 μM $^{14}$C-phenobarbital and 0, 0.5 or 1.0 ml of cytosol prepared from the livers of untreated rats. Reaction mixtures incubated for 60 minutes at 4° as well as reaction mixtures containing no cytosol and incubated for 60 minutes at 37°
were used as controls. The uptake of radioactivity into nuclei was determined after the nuclei from each reaction flask were isolated by centrifugation. Therefore, this method will not distinguish between radioactivity outside of the nucleus associated with the nuclear membrane and radioactivity which is inside the nucleus and not associated with the nuclear membrane.

The uptake of phenobarbital by nuclei was enhanced during incubation at 37° over nuclei incubated at 4°. This enhanced uptake during incubation was further enhanced by the addition of cytosol, Figure 9. There was an apparent linear relationship between the amount of phenobarbital taken up by nuclei and the number of nuclei in the incubation flask, and this line could be extrapolated through the origin. From the slopes of the lines the concentration of the drug in nuclei was determined. For incubations containing no cytosol, 0.5 or 1.0 ml cytosol (equivalent to 0, 125 or 250 mg of liver) the concentration of phenobarbital was 4, 12 and 20 ng phenobarbital per 10^8 nuclei, respectively.

The influence of adding cytosol to the incubations of nuclei was determined by subtracting the concentration of phenobarbital in nuclei incubated without cytosol from the concentrations obtained when either 0.5 or 1.0 ml of cytosol was added. Thus, 0.5 and 1.0 ml cytosol caused an increase in the uptake of phenobarbital by nuclei of 8 and 16 ng/10^8 nuclei. It is interesting to note that doubling the amount of added cytosol from 0.5 to 1.0 ml
Rat liver nuclei were isolated by Method II as described in Methods. The concentration of nuclei was determined in an aliquot stained with toluidine blue using light microscopy and a hemacytometer. Each 50 ml Erlenmeyer flask contained the following components in a total volume of 3 ml:

- 4 mM MgCl$_2$
- 8 mM 2-mercaptoethanol
- 16 mM KCl
- 2 mM ATP
- 5 mM D-glucose
- 0.1 M Tris-HCl buffer, pH 7.4
- 90 μM $^{14}$C-phenobarbital (3.1 mCi/mole)

The reaction mixture was incubated for 60 minutes at 37°. Controls were incubated for 60 minutes at 4° or boiled and incubated for 60 minutes at 37°. Control values were subtracted from each value. Reactions were terminated by plunging the flasks into ice. Nuclei were reisolated by centrifugation through sucrose, and the radioactivity was determined in the nuclear pellet.
caused a doubling of the amount of phenobarbital taken up by nuclei from 8 to 16 ng/10^8 nuclei. The saturability of adding cytosol to stimulate the uptake of phenobarbital could not be tested because the volume of the incubation medium was limited to 3 ml.

The uptake of phenobarbital by isolated nuclei was compared to the concentration of radioactivity, expressed as ng equivalents of phenobarbital/g of liver, taken up by liver nuclei after \textit{in vivo} administration of the compound, Table 4. The maximum concentration of phenobarbital taken up by isolated nuclei was 23 ng/g of liver, while the concentration of phenobarbital in isolated nuclei from rats treated with 80 mg/kg was 30 ng/g of liver. Thus, good agreement was found between \textit{in vitro} and \textit{in vivo} concentrations of phenobarbital in rat liver nuclei.

\section*{The Effect of Phenobarbital Treatment on Rat Liver Polyamines}

Some of the earliest events in rat liver cells after phenobarbital treatment occur within minutes after administration. However, these events have not been directly linked to an anabolic effect of the drug which occurs after repeated administration. The content of the polyamines putrescine, spermidine and spermine in rat liver have been implicated as compounds which are rapidly synthesized under conditions of rapid liver growth (Russell, 1973). Therefore, it was postulated that since phenobarbital treatment causes biochemical changes in the liver soon after injection, it may also cause an accumulation of one or all of the polyamines
TABLE 4

COMPARISON OF **IN VIVO** AND **IN VITRO**
UPTAKE OF PHENOBARBITAL BY RAT LIVER NUCLEI

<table>
<thead>
<tr>
<th>Phenobarbital, ng/gram of liver</th>
<th>ml Cytosol Added</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>in vitro</strong></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
</tr>
<tr>
<td>23</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>in vivo</strong></td>
<td>30</td>
</tr>
</tbody>
</table>

a. The concentration of phenobarbital in isolated nuclei after in vitro incubation was calculated from the data in Figure 9.

b. Cytosol prepared from untreated rats was added to incubation flasks as described in Methods. One ml cytosol was equivalent to 250 mg of liver.

c. The in vivo phenobarbital concentration in liver nuclei from treated rats, 80 mg/kg, was calculated from the data in Figure 3.
prior to measurable anabolism in the liver. To examine this possibility the effect of phenobarbital treatment on the concentration of liver polyamines was examined as a function of time.

Rats which were injected intraperitoneally with saline and uninjected controls did not have significantly different concentrations of putrescine, spermidine, or spermine and were combined to form the control group. In this control group the concentrations of putrescine, spermidine, and spermine were 75 ± 22, 952 ± 104 and 842 ± 107 μmole/gram of liver (mean ± S.D.), respectively.

Several experiments were conducted to investigate possible changes in polyamine content after phenobarbital treatment. In separate experiments the polyamines were determined by either amido black or ninhydrin procedures as described in Methods. These studies resulted in similar patterns.

The polyamine concentration in rat liver after 2 hours of phenobarbital treatment, 80 mg/kg, had changed in the following manner: putrescine and spermine decreased from mean values of 75 and 842 μmole/gram to 67 and 672 μmole/gram, respectively, while the concentration of spermidine increased from 952 to 1085 μmole/gram, Figure 10. These changes in polyamine concentration were also reflected in the ratios of spermidine/spermine and putrescine/spermidine, Figure 11. The former ratio increased from 1.10 to 1.59 while the latter ratio decreased from 0.077 to 0.062. These changes at 2 hours are consistent with the hypothesis that
phenobarbital treatment causes an increase in the concentration of spermidine at the expense of both putrescine and spermine. The pattern of increased spermidine concentration and decreased spermine as well as putrescine concentrations was consistent in each of the six rats examined after two hours of phenobarbital treatment. It was also noted that the concentrations of the polyamines showed more variability between animals than the ratios, which showed more consistency from animal to animal.

The concentration of spermidine in the livers of rats treated with phenobarbital continued to rise until 8 hours after treatment. All of the polyamines decreased in concentration at 12 hours to control levels or less. The concentration of spermidine was elevated above the control level at each time examined from 16 to 36 hours after treatment. The concentrations of putrescine and spermine were below control levels 36 hours after treatment, while the concentration of spermidine was elevated at that time. The ratio spermidine/spermine, Figure 11, increased steadily beyond 12 hours, while the ratio putrescine/spermidine beyond 12 hours initially increased and then decreased after 20 hours.

The pattern of the concentrations of the polyamines and their ratios in rats liver after treatment with phenobarbital, Figures 10 and 11, suggest that phenobarbital causes changes which may be dependent on diurnal variations, but the net result is an increase in the concentration of
FIGURE 10

The Effect of Phenobarbital Treatment on The Polyamine Content of Rat Liver

Rats were injected with 80 mg phenobarbital/kg ip or saline and killed in groups of three. The concentration of each polyamine in rat liver was determined by the ninhydrin method as described in Methods. Each point is the average of duplicate measurements of each polyamine in three rats. In another experiment using groups of three rats in the same time course, but determining the polyamines by the amido black method (see Methods), the pattern was identical.
FIGURE 10

Putrescine

Spermidine

Spermine

Hours After Phenobarbital Treatment
FIGURE 11

The Effect of Phenobarbital Treatment on Polyamine Ratios of Rat Liver

See the legend to Figure 10 for details. The ratios were determined from the concentrations of the respective polyamines.
spermidine to 126% of the control value. Thus, it is seen from the data that phenobarbital does cause an alteration in liver polyamine content, and furthermore, these alterations are consistent with a change in the metabolic pathway favoring the formation of spermidine with a concomitant decrease in the concentrations of its precursors.

The Effect of Phenobarbital Treatment on the Activity of Liver Ornithine Decarboxylase

The changes observed in the concentrations of rat liver polyamines after phenobarbital treatment suggested that phenobarbital treatment may have an effect on ornithine decarboxylase (ODC), which controls the rate limiting step for polyamine synthesis.

Rats were treated with 80 mg phenobarbital/kg intraperitoneally and killed at various times. The substrate for the in vitro reaction was D,L-1-14C-ornithine, and the product measured was evolved 14CO₂. It was assumed for making calculations that only the L-isomer was a substrate for the reaction. Similar results were obtained using either the 20,000 x g or the 100,000 x g supernatant fraction of liver homogenates as the source of the enzyme. The velocity of the reaction was found to be dependent on the concentration of the substrate added to the reaction mixture, and since some ornithine may be present in the enzyme preparation, a saturating amount of substrate was used.

In order to evaluate changes in the specific activity of ornithine in the reaction mixture due to various amounts
of unlabeled endogenous ornithine in the enzyme preparation, two approaches were used. First, the 20,000 x g supernatant fraction was dialyzed for 5 hours against 100 volumes of the medium used for homogenization of the livers. The dialysate was then assayed for ODC activity. Secondly, the enzyme preparation was preincubated for either 15 or 30 minutes before the radiolabeled substrate was added. Neither of these procedures significantly changed the activity of ODC in livers from control or phenobarbital treated animals. Initial studies showed that the addition of phenobarbital to the reaction mixture had no direct effect on ODC activity.

The velocity of the reaction catalyzed by ODC was measured using the 20,000 x g rat liver supernatant fraction as the source of enzyme, Table 5. Substrate concentrations between 10 and 50 μM were apparently too low to obtain linear kinetics, i.e., the substrate was depleted during the 60 minute incubation. However, when the substrate concentration was increased to 1,000 μM and greater, the velocity increased and was apparently near saturation since further increases in substrate concentration did not result in proportional increases in reaction velocity. Therefore, a substrate concentration of 1,000 μM was used to assay for ODC activity in control as well as treated rats.
TABLE 5

KINETICS OF ORNITHINE DECARBOXYLASE ACTIVITY

<table>
<thead>
<tr>
<th>Velocity, mumoles/60 min/gram liver</th>
<th>L-ornithine Concentration, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>10</td>
</tr>
<tr>
<td>7.8</td>
<td>20</td>
</tr>
<tr>
<td>8.0</td>
<td>40</td>
</tr>
<tr>
<td>7.6</td>
<td>50(^b)</td>
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<tr>
<td>72.0</td>
<td>1000</td>
</tr>
<tr>
<td>103.2</td>
<td>3000</td>
</tr>
<tr>
<td>120.3</td>
<td>3500</td>
</tr>
</tbody>
</table>

a. Values are means of at least 3 determinations, each of which is within 15% of the mean. The assay conditions are described in Methods. The reaction velocity is expressed as mumoles \(^{14}\text{CO}_2\) produced per hour per gram of liver.

b. Sham operated rats; reported by Russel and Snyder, 1968.
A representative time course of rat liver ODC activity after phenobarbital treatment is shown in Figure 12. The activity of ODC was increased 190% of controls 10 minutes after treatment with phenobarbital. The activity of ODC in livers of treated rats at 7 and 16 hours was 290 and 300% of the activity in livers of control rats, respectively. The activity of ODC remained elevated compared to the activity in livers of control animals for 24 hours, at which time the activity was 141% of controls.

The stimulation of the activity of ODC after phenobarbital treatment to 2 or 3-fold the activity in livers of untreated rats suggests that at least part of the cause of alterations in the concentrations of polyamines may be due to an increased synthesis of putrescine from ornithine. Putrescine may then be further metabolized to spermidine. However, this data must be interpreted carefully, since putrescine is not only a product of ODC activity but also is a feedback inhibitor of ODC (Tabor and Tabor, 1964). Thus, the stimulation of ODC activity may be due to a decrease in the concentration of putrescine, thereby stimulating ODC indirectly by reducing its inhibition.
Rats were treated with 80 mg phenobarbital/kg ip or an equal volume of saline. Preparation of the 20,000 x g enzyme fraction is described in Methods. Each incubation was carried out in a 10 ml Erlenmeyer flask equipped with a rubber stopper and polyethylene center well containing a 2:1 mixture of ethanolamine and 2-methoxy-ethanol. Incubation mixtures contained in a total volume of 2 ml:

- 0.1 μmole pyridoxal phosphate
- 0.5 μCi DL-ornithine-1-14C (11.0 mCi/mmol)
- 2.0 μmoles L-ornithine
- 0.05 M phosphate buffer, pH 7.2
- 2.5 μmoles dithiothreitol
- 0.5 ml enzyme preparation

The reaction was conducted at 37° for 60 minutes and terminated by addition of 0.5 ml 5N H2SO4. Center wells were counted in an ethanol-toluene phosphor cocktail at 40% efficiency. A heated enzyme blank was subtracted from each value. Each point in the figure is the average of triplicate determinations.
FIGURE 12

Hours After Phenobarbital Treatment

Percent Control

0.16 1 6 7 16 24
DISCUSSION

Repeated injections of high doses of phenobarbital to rats cause an increased activity of certain drug metabolizing enzymes in the liver (Conney, 1967). The results of the studies reported herein indicate that biochemical events occur within minutes after injection of a single dose of the drug. It is suggested that these early events may be related to the ultimate anabolic effect of the drug on the liver.

Although it may be expected that the initial event produced by phenobarbital would be the result of a direct combination of the drug with a receptor, no direct effect of phenobarbital could be demonstrated by the addition of the drug in vitro to liver preparations. The combination of radioactivity from injected $^3$H-phenobarbital with peak I protein from liver cytosol, Figures 5 and 6, could not be demonstrated when the drug was added in vitro, Figure 7. It is possible that the radioactivity bound in peak I was not due to the parent compound, but was due to binding of a metabolite of phenobarbital. However, the binding of radioactivity to peak I protein could not be enhanced in vitro by incubation of liver cytosol and the drug in a drug metabolizing system. This procedure was used by
Bresnik (1966) to demonstrate that a metabolite of 3-methylcholanthrene was bound to protein in the rat liver cytosol. It may be postulated that a minor metabolite of phenobarbital is formed \textit{in vivo} but not \textit{in vitro}, and this metabolite binds to protein in peak I. An alternate possibility is that a protein in peak I which binds phenobarbital is not present prior to phenobarbital treatment. This protein could be altered by drug treatment, or translocated to the liver from the blood or other tissue.

Based on evidence obtained from incubations of isolated nuclei, drug and cytosol which demonstrated that cytosol enhanced the uptake of phenobarbital into nuclei, Figure 9, it is unlikely that the protein associated with the drug in peak I is identical with the factor present in the cytoplasm which enhanced the uptake of the drug into nuclei. That is, the radioactivity associated with peak I protein was only observed after \textit{in vivo} administration of the drug, while cytosol added \textit{in vitro} to isolated nuclei and drug enhanced the uptake of the drug into nuclei. An hypothesis which is consistent with the results of both experiments is that phenobarbital treatment causes the nucleus to become "leaky", thus allowing the uptake of phenobarbital itself as well as the drug-protein complex of peak I. The nuclei from the livers of rats treated for 20 minutes with phenobarbital contained more radioactivity bound to protein in the nucleoplasm than nuclei of rats treated for 5 minutes, Figure 8.
The binding of radioactivity to protein of the nucleoplasm at 20 minutes, however, was diffuse, and the appearance of peak I binding in the nucleus could not be demonstrated.

The stimulation of the activity of RNA polymerase I five minutes after treatment of rats with phenobarbital, Figure 4, occurred when binding to protein in peak I of the cytosol was high and nucleoplasmic binding was low, Figures 5, 6 and 8. Since most of the radioactivity in the nucleoplasm at 5 minutes was apparently in the free form, it was postulated that phenobarbital itself may have caused a direct stimulation of the polymerase I enzyme. However, addition of phenobarbital, from \(10^{-7}\) to \(10^{-5}\) M, had no effect or was slightly inhibitory to the activity of RNA polymerase I, Table 2, and was slightly inhibitory to RNA polymerase II, Table 3. Therefore, it was concluded that stimulation of RNA polymerase I by phenobarbital required a factor in addition to the drug itself.

Further studies on RNA polymerase I activity measured in vitro revealed that the addition of liver cytosol in combination with phenobarbital or the addition of cytosol alone caused a stimulation of about the same magnitude as after in vivo administration of the drug, Table 2 and Figure 4. The addition of cytosol equivalent to either 10 or 100 mg of liver and in the absence of drug caused stimulation to 148 and 300% of control activity. The addition of phenobarbital in concentrations from \(10^{-7}\) to \(10^{-5}\) M had no effect on the enzyme activity in addition to the stimulation caused
by the addition of cytosol alone. The stimulation of RNA polymerase I activity by the cytosol fraction was interpreted in conjunction with the results of experiments which examined the uptake of phenobarbital by isolated nuclei in the presence of cytosol, Figure 9. From the latter experiments it was concluded that phenobarbital caused the nuclear membrane to become "leaky" or more penetrable by phenobarbital as well as other components of the cytosol. An increased penetrability of the nucleus by a factor in the cytosol which stimulates the activity of RNA polymerase I is consistent with the hypothesis that this factor in the cytosol, possibly a protein or amino acid, under control conditions is excluded from the nucleus, but upon phenobarbital treatment enters the nucleus. Stimulation of the activity of RNA polymerase I in isolated nuclei has been observed by increasing the concentration of amino acids in an in vitro incubation medium (Franze-Fernandez and Fontanive-Senguesa, 1973). The addition of inhibitors of protein synthesis blocked the stimulation of RNA polymerase I, indicating that protein synthesis was involved in the process. In order for the hypothesis to be consistent with the in vitro stimulation of RNA polymerase I activity by the addition of cytosol in the absence of phenobarbital, it must be assumed that isolated rat liver nuclei under conditions of the incubation at 37° become penetrable by the stimulatory factor even in the absence of the drug.
If a factor is present in liver cytosol which enters the nucleus upon phenobarbital treatment, then this factor may become reduced in concentration or depleted by prior drug treatment. To test this possibility, cytosol was isolated from rats pretreated with phenobarbital for either 5 or 30 minutes. The pretreated cytosol was added to isolated nuclei from untreated rats, and the activity of RNA polymerase I was measured, Table 2. Pretreated cytosol was less effective in stimulating RNA polymerase I activity than cytosol from untreated rats. The same reduction in the stimulative activity of cytosol by pretreatment with phenobarbital was observed when $10^{-5}$M phenobarbital was added to the incubation medium. These results support the contention that a factor in the cytosol can enter the nucleus and stimulate RNA polymerase I activity, and this translocation from cytosol to nucleus causes at least partial depletion of the cytosol of this factor.

Gelboin, et al (1967) have suggested that stimulation of genetic activity may cause cytoplasmic alterations which result in induction. This suggestion was based on measurement of increased RNA polymerase I and II activity 20 hours after administration of phenobarbital. Similar results were obtained by Orrenius et al (1967) who measured RNA polymerase I and II activities as a function of time after injection of phenobarbital at 0 and 24 hours. The activity of RNA polymerase I was elevated above controls at about 8 hours and later, while RNA polymerase II activity was not
elevated until after the second injection of the drug. The earliest time examined by these authors was 3 hours. Therefore, the data shown in Figure 4 together with the data of Gelboin et al (1967) and Orrenius et al (1967) indicate that the activity of RNA polymerase I is stimulated several times after a single administration of phenobarbital while the activity of RNA polymerase II is probably not significantly elevated until after repeated injections of the drug.

The stimulation of RNA polymerase I as early as 5 minutes after injection of phenobarbital is the earliest reported change in biochemical activity in the nucleus. Since increases in the synthesis of total rat liver microsomal protein have been observed as early as 1 hour after phenobarbital treatment (Kuriyama et al, 1969), the observation of genetic activity in liver nuclei at times earlier than 1 hour is consistent with a cause-effect relationship.

The sequence of events in rat liver after phenobarbital treatment reported herein are summarized in Table 6. Within the first 10 minutes after administration of phenobarbital, the activities of RNA polymerase I and ODC have increased about 2-fold over control levels. The concentration of the drug has reached peak levels in serum as well as the liver cytoplasm and nuclei. The concentration of radioactivity bound to the microsomal fraction of the liver is increasing. The binding of radioactivity to protein
### Sequence of Events in Rat Liver After Treatment with Phenobarbital

<table>
<thead>
<tr>
<th>Time, Minutes</th>
<th>Event</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Drug reaches highest concentration in nuclei</td>
<td>Fig. 3</td>
</tr>
<tr>
<td>5</td>
<td>Polymerase I stimulated to 220% of control</td>
<td>Fig. 4</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic binding of drug high in vivo</td>
<td>Fig. 5</td>
</tr>
<tr>
<td></td>
<td>Nucleoplasmic binding of drug low in vivo</td>
<td>Fig. 8</td>
</tr>
<tr>
<td>10</td>
<td>Drug concentration peaks in cytoplasm and serum</td>
<td>Fig. 2</td>
</tr>
<tr>
<td></td>
<td>ODC activity doubles</td>
<td>Fig. 12</td>
</tr>
<tr>
<td>20</td>
<td>Cytoplasmic binding of drug low</td>
<td>Fig. 6</td>
</tr>
<tr>
<td></td>
<td>Nucleoplasmic binding of drug high</td>
<td>Fig. 8</td>
</tr>
<tr>
<td>30</td>
<td>Second peak of polymerase I activity</td>
<td>Fig. 4</td>
</tr>
<tr>
<td>60</td>
<td>Polymerase activity near control level</td>
<td>Fig. 4</td>
</tr>
<tr>
<td>120</td>
<td>Spermidine/Spermine ratio 160% of control</td>
<td>Fig. 11</td>
</tr>
<tr>
<td></td>
<td>Putrescine/Spermidine ratio 70% of control</td>
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eluting in peak I (high molecular weight fraction) from gel filtration of cytosol was greater at 5 minutes than at 20 minutes. At 20 minutes the radioactivity in nucleoplasm was bound to a disperse fraction of protein, while at 5 minutes most of the radioactivity was not bound. At 30 minutes RNA polymerase I activity increased after returning to near control level. At 2 hours the concentration of liver spermidine was elevated, apparently at the expense of putrescine and spermine.

It is concluded that some of the earliest effects of phenobarbital in rat liver are related to uptake of the drug by nuclei. The uptake of drug into nuclei may be accompanied by movement of a cytoplasmic factor into the nucleus resulting in stimulation of RNA polymerase I. Thus, the first RNA synthesis to be stimulated by phenobarbital treatment may be ribosomal-like rather than messenger-like RNA.
SUMMARY

Studies were conducted to elucidate some of the very early biochemical changes in rat liver following a single injection of 80 mg phenobarbital/kg. The focus of the experiments was on changes in the hepatocyte cytoplasm and nucleus which may be expected to lead to an anabolic change (induction) in the liver due to the drug. The results, conclusions, and implications of these studies are summarized as follows: 1) the distribution of radioactivity after a single dose of 80 mg $^3$H-phenobarbital/kg injected intraperitoneally was determined as a function of time in serum as well as liver cytosol, microsomes and nuclei. The peak concentrations of radioactivity in serum and cytosol were at approximately 10 minutes, and were between 160 and 180 µg/g. The apparent half life and volume of distribution were 9.9 hours and 480 ml/kg, respectively. The peak concentration in nuclei was 16 ng/mg of nuclear protein at 1 minute. The concentration in washed microsomes increased with time up to 2 hours, when a concentration of 180 ng/mg of microsomal protein was obtained; 2) within one hour after phenobarbital treatment of rats the activity of DNA-dependent RNA polymerase I, which synthesizes ribosome-like RNA in the nucleolus,
was stimulated above control activity two times. First, at 5 minutes the stimulation was to 220% of control activity, and secondly, at 30 minutes to 180% of control activity. The activity of RNA polymerase II, which synthesizes messenger-like RNA in the nucleus, was not changed during the first hour following drug treatment. Thus, the earliest biochemical change observed in these studies was a stimulation of activity in the liver nucleus which may lead to increased production of ribosomal RNA; 3) the stimulation of RNA polymerase I activity observed in vivo (see 2 above) was essentially duplicated in vitro when liver cytosol was added to the assay, but not when phenobarbital alone was added. The in vitro stimulation by liver cytosol was to 300% of control, whereas the in vivo stimulation by phenobarbital injection was to 220% of control. Neither phenobarbital nor cytosol added in vitro stimulated RNA polymerase II activity, and may in fact have been slightly inhibitory. The stimulation of RNA polymerase I activity by cytosol was interpreted as support for the hypothesis that liver cytosol contains a factor which stimulates RNA polymerase I upon entering the nucleus. This factor may penetrate the nucleus in vivo in the presence of phenobarbital, but in vitro the nuclei are apparently "leaky" and permit the uptake of the factor into the nucleus in the absence of drug. In support of this hypothesis liver cytosol from phenobarbital treated rats was less stimulative in vitro than cytosol from untreated rats, suggesting that in treated rats this factor may be partially depleted by movement into the nucleus; 4) the uptake of radiolabeled phenobarbital
was determined in vitro using isolated rat liver nuclei incubated in the presence or absence of added liver cytosol. Radioactivity was taken up by nuclei in the absence of cytosol, but the presence of cytosol enhanced this uptake. For incubations containing no cytosol or cytosol equivalent to 125 or 250 mg of liver, the concentrations of phenobarbital taken up by nuclei were 4, 12 and 20 ng per $10^8$ nuclei, respectively. After converting the concentrations to ng/gram of liver, it was found that the maximum uptake in vitro was 23 ng/g, and in vivo following 80 mg/kg the concentration of drug in nuclei was 30 ng/g of liver. Thus, good agreement between in vivo and in vitro uptake of drug by liver nuclei was found. The uptake of drug by nuclei in vitro in the presence of cytosol supports the hypothesis that a factor in the cytosol may combine with the drug and transport into the nucleus; 5) Cytosol isolated from livers of rats injected with $^3$H-phenobarbital and killed at 5 and 20 minutes contained a protein fraction which weakly bound the drug. The extent of this binding was greater at 5 minutes than at 20 minutes. A reciprocal relationship of the cytosol binding was found in nuclei isolated from the same rat livers. The fraction of the drug bound was less than 0.5%, and drug added in vitro was not bound. In vitro drug metabolism did not enhance the binding. It was speculated that the protein of liver cytosol which bound the drug could be the factor which stimulates RNA polymerase I, however, no experiment evidence supports this hypothesis;
6) Administration of 80 mg phenobarbital/kg to rats caused an increase in the activity of ornithine decarboxylase (ODC). The activity of ODC reached a maximum of 300% of control at 16 hours and decreased to near the control level by 24 hours. Early changes in the concentrations of polyamines resulted in an increase of liver spermidine at the expense of both putrescine and spermine. The changes in ODC activity and in the concentration of the polyamines were small compared to changes evoked by partial hepatectomy or other models of rapid liver growth; 7) It was concluded that the earliest change in rat liver biochemistry may be the stimulation of the synthesis of ribosomal RNA. An hypothesis is presented which describes the role of a postulated cytoplasmic factor which may bind to the drug and gain access to the nucleus. Once in the nucleus this factor, either in the presence or absence of drug, may stimulate RNA polymerase I.
APPENDIX

The Preparation of Nuclei Isolated by Method I for Electron Microscopy

The final pellet from the nuclear isolation described in Methods (Method I) was fixed in cold 6% glutaraldehyde with phosphate buffer, pH 7.4; post fixed in cold 1% osmium tetroxide with phosphate buffer, pH 7.4; and dehydrated in graded ethyl alcohol (50%, 70%, 95%, absolute). Nuclei were then embedded in Spurr Low Viscosity Embedding Medium, and sections cut 600-900 Å with glass knives. Staining was with uranyl acetate and lead citrate. Sections were viewed with a Phillips 300 electron microscope.
BIBLIOGRAPHY


