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# EARLY EFFECTS OF PHENOBARBITAL ON THE ADENINE NUCLEOTIDE POOL OF RAT LIVER

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**Abstract**—The effect of a single dose of phenobarbital (80 mg/kg, i.p.) on the concentrations of ATP, ADP and AMP and on the synthesis of ATP from $^{32}Pi$ were examined. Neither treatment with phenobarbital nor intraperitoneal injection of saline had a major effect on the steady state levels of the three nucleotides within 24 hr. The synthesis of ATP after treatment with either phenobarbital or saline was elevated at about 6 hr. This effect was presumably the result of stress due to intraperitoneal injection. A larger increase in ATP synthesis occurred in phenobarbital-treated rats at 14 hr after treatment and persisted throughout 24 hr. This peak was absent in saline-treated rats and is interpreted to be related to the anabolic effect of phenobarbital in rat liver.

ASIDE from its sedative action, phenobarbital has pronounced effects on hepatic metabolism. These alterations have been well documented and include extensive proliferation of the smooth endoplasmic reticulum, elevated levels of certain microsomal enzymes and an increase in gross liver weight.[1] Although a number of studies have implicated the *de novo* synthesis of proteins in the hepatic responses to phenobarbital,[2–5] the mechanism of this response is not clear.

Experiments described in this report were directed toward an investigation of the effects of phenobarbital on cellular energetics, in particular, the effects on the adenine nucleotides. Recent investigations by Atkinson *et al.*[6–8] and Brenner *et al.*[9] suggest that alterations in the steady state concentrations of the adenine nucleotides may be important in the anabolic response of cells to agents like phenobarbital. Alternatively, it has been observed that in regenerating liver, another form of experimental hepatic growth, the turnover of ATP is markedly increased early in response to surgery.[10] It seemed possible that phenobarbital might similarly effect ATP turnover and that this response might be related to the drug-induced hepatic growth. With these observations in mind, the steady state concentrations of ATP, ADP and AMP and the incorporation of $^{32}P$-orthophosphate into ATP were measured within 24 hr after a single dose of phenobarbital.

## MATERIALS AND METHODS

Male Wistar rats (100–150 g) were fasted 12 hr before the experiment, and food was withheld throughout the experiment. Animals were injected intraperitoneally with either 80 mg/kg of sodium phenobarbital (Intra Products, Dayton, Ohio) or an equal volume of pyrogen-free sterile saline (Travenol Laboratories Inc., Morton Grove, Ill.).

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In some instances animals were not injected and retained as untreated controls. At various times up to 24 hr thereafter, animals were anesthetized with a mixture of diethyl ether and 100% oxygen as described by Bucher and Swaffield,[11] and the various manipulations described below were performed. In experiments in which the incorporation of radioactive phosphate into adenine nucleotide phosphate was estimated, 10 $\mu$c of neutral pyrophosphate-free sodium $^{32}$P-orthophosphate was injected in 0·1 ml of sterile saline (Travenol) by tail vein. Livers were sampled *in situ* 5 min later. In experiments in which adenine nucleotide content was measured separately, liver samples were also taken 5 min after induction of anaesthesia.

*In situ* sampling was performed by clamping thin wafers of the left lateral hepatic lobe between stainless steel plates (1 × 10 × 25 mm) that had been silver-soldered to the ends of a hemostat. The plates were pre-cooled in liquid nitrogen before sampling, and samples were immediately plunged into liquid nitrogen. Subsequently, the tissue was rapidly weighed in the cold and then immediately homogenized in 5% PCA in conical ground glass homogenizers. The clear yellow supernate was collected by centrifugation, and used to determine the concentrations of ATP, ADP and AMP and the specific radioactivity of the adenine nucleotide phosphate.

The incorporation of radioactive phosphate into adenine nucleotides was estimated essentially as described by Ove *et al.*[10] Briefly, aliquots of the 5 per cent PCA supernate from homogenates were mixed with acid-washed Norite A and allowed to stand on ice for 5 min. The charcoal was washed repeatedly with cold distilled water. The uptake of $^{32}$P-phosphate not incorporated into adenine nucleotide was estimated by counting the radioactivity in these washes. These data are expressed as the amount of radioactivity per micromole of inorganic phosphate in the aqueous eluates. The washed charcoal was resuspended in HCl, boiled for 10 min and the supernate was collected. Neutralized aliquots of the HCl supernatant fluid were used for determination of adenine nucleotide phosphate radioactivity by liquid scintillation in Bray's solution[12] and for determinations of phosphate.[13] Data are expressed as counts/min/micromole adenine nucleotide phosphate.

Fluorometric methods described by Lowry *et al.*[14] were used for the determination of ATP, ADP and AMP in aliquots of the 5% PCA supernatant of the liver homogenate. This supernatant was neutralized with Tris buffered-KOH and the potassium perchlorate precipitate was removed before assay. Data are expressed as micromoles per gram of wet weight.

## RESULTS

The effect of a single intraperitoneal injection of either phenobarbital (80 mg/kg) or an equal volume of saline (0·1 ml) on the concentrations of ATP, ADP and AMP was estimated before injection and at 6, 14, and 24 hr after injection. These data are expressed in Table 1. Neither treatment altered the concentrations of ATP or ADP significantly from the corresponding value for untreated animals; nor were the ATP or ADP concentrations from phenobarbital- and saline-treated rats different from each other at any time interval. The concentration of AMP varied more widely than the ATP and ADP concentrations. Intraperitoneal administration of saline or phenobarbital raised the levels of AMP at some time periods, and at 6 and 14 hr there were significant differences between barbiturate- and saline-treated animals. These changes

TABLE 1. EFFECT OF PHENOBARBITAL ON THE ADENINE NUCLEOTIDE CONTENT OF RAT LIVER*

| Treatment | ATP (μmoles/g) | ADP (μmoles/g) | AMP (μmoles/g) | Total adenine nucleotides (μmoles/g) |
|---|---|---|---|---|
| Untreated | 2·14 ± 0·069 | 0·87 ± 0·044 | 0·17 ± 0·010 | 3·17 ± 0·112 |
| Phenobarbital 6 hr | 2·42 ± 0·137 | 0·73 ± 0·100 | 0·29 ± 0·026†‡ | 3·43 ± 0·224 |
| Saline 6 hr | 1·86 ± 0·199 | 0·99 ± 0·042 | 0·47 ± 0·026† | 3·32 ± 0·198 |
| Phenobarbital 14 hr | 1·94 ± 0·080 | 0·77 ± 0·036 | 0·16 ± 0·005‡ | 2·78 ± 0·099 |
| Saline 14 hr | 2·00 ± 0·037 | 0·83 ± 0·026 | 0·46 ± 0·039† | 3·29 ± 0·036 |
| Phenobarbital 24 hr | 1·92 ± 0·135 | 0·77 ± 0·082 | 0·34 ± 0·046† | 3·04 ± 0·204 |
| Saline 24 hr | 2·05 ± 0·126 | 0·85 ± 0·096 | 0·23 ± 0·046 | 3·13 ± 0·246 |

* Rats were injected intraperitoneally with 0·1 ml of a solution of phenobarbital (80 mg/kg) or an equivalent volume of saline, and killed under anesthesia at the indicated times. Adenine nucleotides were determined as described in Methods. Each value is the mean ± S.E.M. of five animals. Data are expressed as micromoles per gram of wet weight. Levels of significance were determined by Student's *t*-test.

† Different from untreated animals to at least $P < 0·05$.

‡ Different from corresponding saline-treated animals to at least $P < 0·05$.

in AMP concentration did not appear in a consistent pattern, and it would appear that although the administration of agents by the intraperitoneal route may have effects on the concentrations of AMP, the data in Table 1 do not suggest that phenobarbital causes any major alteration in the adenine nucleotide concentration of rat liver at the times investigated.

TABLE 2. EFFECT OF PHENOBARBITAL ON THE UPTAKE OF RADIOACTIVE PHOSPHATE BY RAT LIVER*

| Treatment | cpm/μmole Pi |
|---|---|
| Untreated | 33,900 ± 5550 |
| Phenobarbital 6 hr | 39,100 ± 5450 |
| Saline 6 hr | 31,200 ± 4100 |
| Phenobarbital 6 hr | 33,100 ± 2910 |
| Saline 6 hr | 36,700 ± 3440 |

* Rats were injected with either 80 mg/kg of phenobarbital or an equivalent volume of saline (0·1 ml), and then at the indicated times the animals were injected by tail vein with 10 μc $^{32}$-orthophosphate as described in Methods. Five min later the liver was sampled and non-nucleotide radioactivity and inorganic phosphate were determined as described in Methods. Data are expressed as counts/min/micromoles of phosphate; each value is the mean ± S.E.M. of five animals.

The rate of incorporation of $^{32}$P-orthophosphate into nucleotide phosphate was estimated before and at various intervals up to 24 hr after either phenobarbital or saline had been administered by the intraperitoneal route (Fig. 1). In these experi-

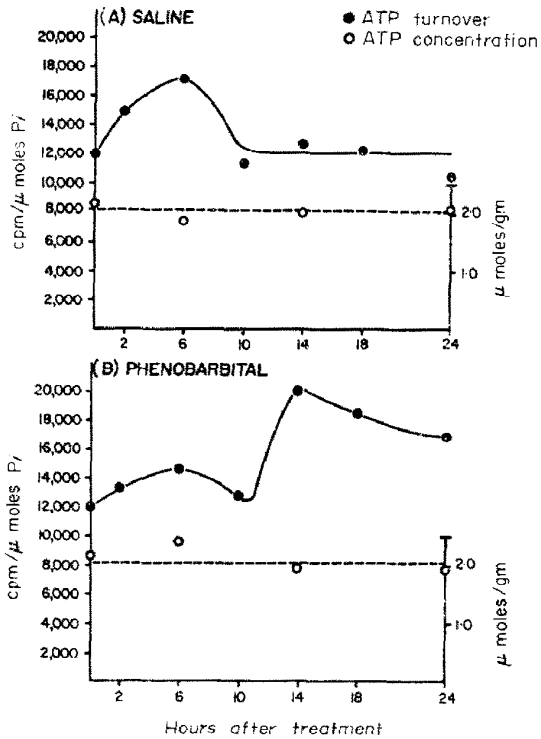


FIG. 1. Effect of phenobarbital and saline on ATP content and turnover. Rats were injected with saline (A) or phenobarbital (B) as described in Table 1, and at the indicated times were pulse-labeled with 10 $\mu$c $^{32}$P-orthophosphate for 5 min under ether anesthesia. Points at zero time represent untreated animals. Liver samples were removed and incorporation of radioactivity into adenine nucleotides was determined as described in Methods. Data are expressed as counts/min/micromole of adenine nucleotide phosphate. The concentrations of ATP at various time intervals from Table 1 are also shown.

ments, animals were injected with 10 $\mu$c $^{32}$P-orthophosphate by tail vein 5 min before they were killed, and the specific activity of nucleotide phosphate was estimated as described in Methods. Since under these conditions about 90 per cent of the radioactivity found in nucleotide phosphate is in phosphate of ATP and since estimates of the radioactivity in non-nucleotide phosphate (Table 2) indicate that neither phenobarbitol nor saline affected the hepatic uptake of radioactive phosphate, these data have been considered to be indicative of ATP synthesis.[10]

As can be seen in Fig. 1A, saline injection by itself caused considerable radioactivity to be incorporated into ATP; the increased rate of ATP synthesis showed a maximum of about 6 hr and had returned to control by 10 hr post treatment. Animals that had been treated with phenobarbital (Fig. 1B) also incorporated radioactivity into ATP with a maximum at 6 hr after treatment, although at a lesser rate that was not significantly different from untreated animals. At 14 hr, however, the rate of incorporation of phosphate into ATP increased dramatically and remained high throughout the 24-hr experimental period. This rate of ATP synthesis in barbiturate-treated animals was significantly different from both untreated and corresponding saline-treated animals.

TABLE 3. EFFECT OF PHENOBARBITAL ON PLASMA CORTICOSTERONE*

| Treatment | Plasma corticosterone ($\mu$g/mg) |
|---|---|
| Phenobarbital 6 hr | 0·063 ± 0·014* |
| Saline 6 hr | 0·064 ± 0·010 |
| Phenobarbital 14 hr | 0·093 ± 0·010† |
| Saline 14 hr | 0·128 ± 0·010 |

* Rats were treated as described in Table 1 and killed at the indicated times by decapitation and bled into heparinized tubes. Corticosterone was determined in the plasma by the method of Moncloa *et al.*[15] Each value is the mean ± S.E.M. of five animals. Data are expressed as micrograms milliliter of plasma.
† $P > 0.05$ by Student's *t*-test.

In light of the increased synthesis of ATP at 6 hr in both barbiturate- and saline-treated animals, the possibility of an adrenal cortical stress response was considered. To estimate the importance of this response, plasma corticosterone was determined at 6 and 14 hr after treatment with phenobarbital or saline (Table 3). The plasma corticosterone levels did not change with either treatment.

## DISCUSSION

Data have been presented that suggest that intraperitoneal injection of neither saline nor phenobarbital has a marked effect on the concentrations of the adenine nucleotides within 24 hr. However, both treatments have substantial effects on the synthesis of ATP in the same time period. The effects of both treatments on the rate of synthesis and the steady state concentrations of ATP are presented in Figs. 1A and 1B. Since the concentration of ATP was not affected by either phenobarbital or saline, the rate of ATP-$^{32}$P synthesis may be considered to be an index of ATP turnover.

The ATP turnover rate in rat liver was markedly elevated at 2 and 6 hr after intraperitoneal injection of a sterile pyrogen-free saline solution, and then returned to normal and remained at this level through 24 hr. Although this effect was unexpected, similar results have been obtained by Ove *et al.*[10] after injection of Celite. The response to Celite can be abolished by adrenalectomy, but cannot be produced by administration of corticoids (except intraperitoneally). Similarly, the saline-induced response reported here is not accompanied by an increase in plasma corticosterone (Table 3) and is presumably caused by another adrenal hormone (possibly epinephrine) in response to the stress of intraperitoneal injection. In rats treated with phenobarbital, a similar although attenuated maximum in ATP turnover was observed at 6 hr after treatment. This maximum was also considered to be a response to intraperitoneal injection. As well as the peak at 6 hr, rats treated with phenobarbital had an increase in the rate of ATP turnover at 14 through 24 hr. This latter increase in ATP turnover is present only in drug-treated animals. Ove *et al.*[10] have observed a similar increase in ATP turnover in regenerating liver.

The second peak in ATP turnover seen in phenobarbital-treated rats is presumably

the result of barbiturate treatment. Although the data reported here do not establish the function of the increased ATP turnover in the anabolic response to phenobarbital, it is interesting that the ATP turnover is well correlated in time with an increase in the appearance of ribosomal RNA in the rough endoplasmic reticulum.[16–19] It seems possible that the appearance of ribosomes and the ATP turnover may be part of a coordinated response to phenobarbital that results in the synthesis of protein or lipid components of the endoplasmic reticulum. Similar correlation between phospholipid components of the endoplasmic reticulum and membrane-bound ribosomes have been observed by Tata[20] in rat liver after the administration of anabolic hormones.

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