MICROBODIES IN EXPERIMENTALLY ALTERED CELLS

II. The Relationship of Microbody Proliferation to Endocrine Glands

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ABSTRACT

The liver cells of intact male rats given ethyl-α-p-chlorophenoxyisobutyrate (CPIB) characteristically show a marked increase in microbodies and in catalase activity, while those of intact female rats do not. In castrated males given estradiol benzoate and CPIB the increase in catalase activity and microbody proliferation is abolished, while in castrated females given testosterone propionate and CPIB the livers show a marked increase in microbodies and in catalase activity. No sex difference in microbody and catalase response is apparent in fetal and neonatal rats. Both sexes show a sharp rise in catalase activity on the day of birth, with a rapid decline at 5 days after birth. Thyroidectomy abolishes the hypolipidemic effect of CPIB in rats, but microbody proliferation and increase in catalase activity persists in thyroidectomized male rats, indicating that microbody proliferation can be independent of hypolipidemia. Adrenalectomy does not alter appreciably the microbody-catalase response to CPIB. These experiments demonstrate that (*1*) in adult rats, hepatic microbody proliferation is dependent to a significant degree upon male sex hormone but is largely independent of thyroid or adrenal gland hormones; (*2*) hepatic microbody proliferation is independent of the hypolipidemic effect of CPIB; (*3*) displacement of thyroxine from serum protein may not be sufficient cause for stimulation of microbody formation.

INTRODUCTION

Previous studies (1, 2) demonstrated a significant increase in number of microbodies (peroxisomes) and concomitant rise in catalase activity in male rat liver following administration of ethyl-α-p-chlorophenoxyisobutyrate (Clofibrate, CPIB), a hypolipidemic drug which lowers serum cholesterol and triglycerides in man (3) and in experimental animals (4, 5). Microbodies are a class of cell organelles in liver and kidney of several species and in the protozoan *T. pyriformis* that have been characterized biochemically by de Duve and coworkers (6) who showed that they possess enzymes necessary for production and destruction of hydrogen peroxide. Leighton et al. recently reported methods for mass isolation of these structures, in fractions of improved purity, from livers of rats injected with Triton WR-1339 (7).

A previous study from our laboratory (2) demonstrated that, in female rats given doses of CPIB identical to those given to male rats for the same duration, there was no conspicuous increase either in microbody number or in catalase activity.

TABLE I

Distribution of Rats for Experiments Relating Microbody Proliferation to Gonadal, Adrenal, and Thyroid Hormones

Group	No. of animals
Males	
1. Intact control	4
2. Intact + CPIB	5
3. Castrated control	5
4. Castrated + CPIB	9
5. Castrated + CPIB + estradiol benzoate (0.2 mg/day; subcutaneous)	9
6. Adrenalectomy	4
7. Adrenalectomy + CPIB	9
8. Thyroidectomy	11
9. Thyroidectomy + CPIB	6
Females	
10. Intact control	4
11. Intact + CPIB	5
12. Castrated control	5
13. Castrated + CPIB	9
14. Castrated + CPIB + testosterone propionate (75 μg/day; subcutaneous)	9
15. Adrenalectomy	4
16. Adrenalectomy + CPIB	4

The primary purpose of the experiments described here was to assess the relationship of the CPIB-induced microbody and catalase response to the hormonal status of rats, especially with reference to sex hormones. In addition, the effect of thyroidectomy on hepatic microbody proliferation was investigated since previous reports (8–10) suggested that one of the mechanisms of action of CPIB was to displace thyroxine from serum protein, with consequent increase in concentration of thyroxine in liver cells. Since thyroidectomy abolishes the hypolipidemic effect of CPIB (8, 11), it was apparent that examination of livers from thyroidectomized rats offered one means of clarifying the specificity of hypolipidemia as a stimulus for microbody proliferation (1, 2, 12). For enlarging the basis for comparison, livers from fetal, newborn, and adrenalectomized rats as well as from rats given other agents that displace thyroxine from serum protein were included in these experiments.

MATERIALS AND METHODS

For experiments designed to study the relationship of microbody proliferation to the effects of thyroid, adrenal, and gonadal hormones, rats of both sexes were distributed as indicated in Table I. Gonadectomized and thyroidectomized Sprague-Dawley rats were obtained from Hormone Assay Laboratories, Chicago, Ill. Bilateral adrenalectomy was performed on male F-344 rats under Metofane (methoxyflurane) anesthesia. All animals weighed between 110 and 130 g. After removal of gonads or thyroid glands, animals were caged individually and placed on the basal diet of Purina Chow and water *ad lib* for 2 wk before addition of CPIB to the regimen. Adrenalectomized animals were treated similarly, but were given 1% NaCl in their drinking water. All animals were weighed weekly and killed after 4 wk on CPIB, at which time the liver was removed, weighed, and separated into samples for morphological and biochemical studies. In groups 5 and 14, estradiol benzoate and testosterone propionate, respectively, were given according to the schedule indicated in Table I.

For studying the effects of other agents that decrease binding of thyroxine to serum proteins, eight male Sprague-Dawley rats (two rats per group) were given 2,4-dichlorophenoxyacetic acid (0.125% in diet), trypan blue (25 mg/kg, subcutaneously), novobiocin (300 mg/kg, i.p.) and acetylsalicylic acid (1% in diet) for 4 wk, at which time the animals were killed, and livers were removed and weighed, and samples were processed for light and electron microscopy.

For a comparison of sex difference in microbody response to CPIB between adult rats and fetal and newborn animals, 20 sperm-positive female Sprague-Dawley rats were given the basal diet containing 0.25% CPIB. For biochemical and morphological examination, five pregnant rats were sacrificed at 20 days of gestation and the livers of litters were pooled according to sex. Livers of newborn and 5-day-old rats were similarly pooled.

Microscopy

From all groups, samples of liver were fixed for 1 hr at 0°C in 2% osmium tetroxide buffered with *s*-collidine or in collidine-buffered glutaraldehyde. The blocks were dehydrated in a graded series of alcohols and embedded in Epon 812. Ultrathin sections were cut with glass knives on an LKB ultramicrotome and stained with lead. Sections were studied in an RCA 3G electron microscope with 100-kv accelerating voltage and a 25-μ objective aperture.

Biochemical Methods

For catalase determinations, 1 g of liver in 4 ml of M/150 phosphate buffer was homogenized at 1–4°C with strokes in a Potter-Elvehjem homogenizer at 400 rpm. Catalase activity was measured by the method of Luck (13) after 24 hr of extraction. For comparing the adequacy and reproducibility of this method to those of alternative methods suggested in the literature, catalase activity in the livers of control male rats was assayed by varying certain procedures and comparing the results to those obtained with the standard assay. It was found that substituting 300 strokes in homogenizing for the usual 20 strokes resulted in only a slightly higher reading of catalase activity. Addition of 1% Triton X-100 to 1 g of liver before 20-stroke homogenization gave results similar to those obtained with the standard method, even when assay was done immediately without extraction for 24 hr.

Total proteins were determined by the Lowry method (14). All determinations were done in duplicate.

RESULTS

Gonadal Hormones

By 4 wk, the livers of intact male rats given CPIB showed the characteristic marked increase in catalase activity compared to that of intact controls ($p > 0.001$) (Fig. 1), and there was a simultaneous increase in the number of microbodies (Fig. 2). Castration alone had no appreciable effect on the number of microbodies or on estimated catalase activity, and castrated males given CPIB showed no significant increase in catalase activity as compared with the catalase activity of castrated or intact controls. Estradiol benzoate given to castrated males receiving CPIB (Fig. 1) was associated with a significantly lower catalase activity than that in all other castrated and/or intact males. The effect of castration and addition of female sex hormones to males is most apparent by comparison, in Fig. 1, of catalase activity in intact rats given CPIB to castrated rats given estradiol benzoate in addition to CPIB ($p > 0.001$). Simultaneously, the number of microbodies in liver cells of castrated males given CPIB and estradiol benzoate did not increase (Fig. 3).

Conversely, intact female rats given CPIB typically showed only a slight increase in catalase activity over that of intact controls ($p > 0.4$) (Fig. 4) and, by electron microscopy, their liver cells had no appreciable increase in number of microbodies (Fig. 5). Daily injections of testosterone propionate to castrated females receiving CPIB, however, were associated with marked increase in catalase activity over that of castrated controls with or without CPIB ($p > 0.001$) (Fig. 4), and, simultaneously, there was a marked increase in the number of hepatic microbodies (Fig. 6).

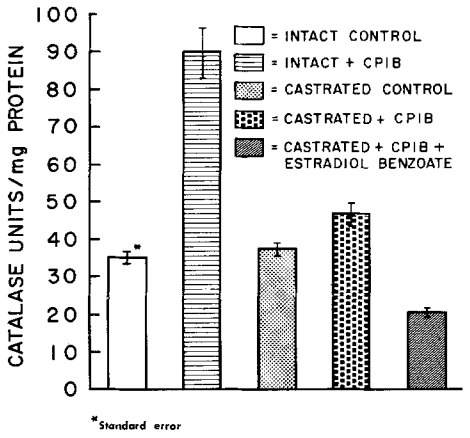

FIGURE 1 Effects of CPIB on liver catalase activity in intact male rats and in castrated male rats given estradiol benzoate.

Thyroidectomy alone did not affect appreciably the catalase activity of male rats consuming only Purina Chow ($p < 0.2$) (Fig. 7). Similarly, thyroidectomy inhibited only slightly the typical elevation of hepatic catalase activity in response to CPIB ($p > 0.01$), and liver cells of these animals had the characteristic increase in the number of microbodies (Fig. 8). Adrenalectomy in male rats (Fig. 9) did not inhibit the effect of CPIB on catalase activity and microbody proliferation ($p > 0.005$). In adrenalectomized females, there was no significant difference in catalase activity between those given only Purina Chow and those given Purina Chow plus CPIB. In both groups, the catalase activity was equal to that in intact females given CPIB.

In fetal rats (20th day of gestation) whose mothers were not given CPIB, the activity of liver catalase was essentially similar in both sexes (Fig. 10). By 5 days after birth, catalase activity increased to the same extent in both sexes and decreased to a slight but parallel degree 5 days after birth. In the livers of fetuses whose mothers received CPIB for the first 20 days of gestation, the catalase activity was elevated in both sexes over

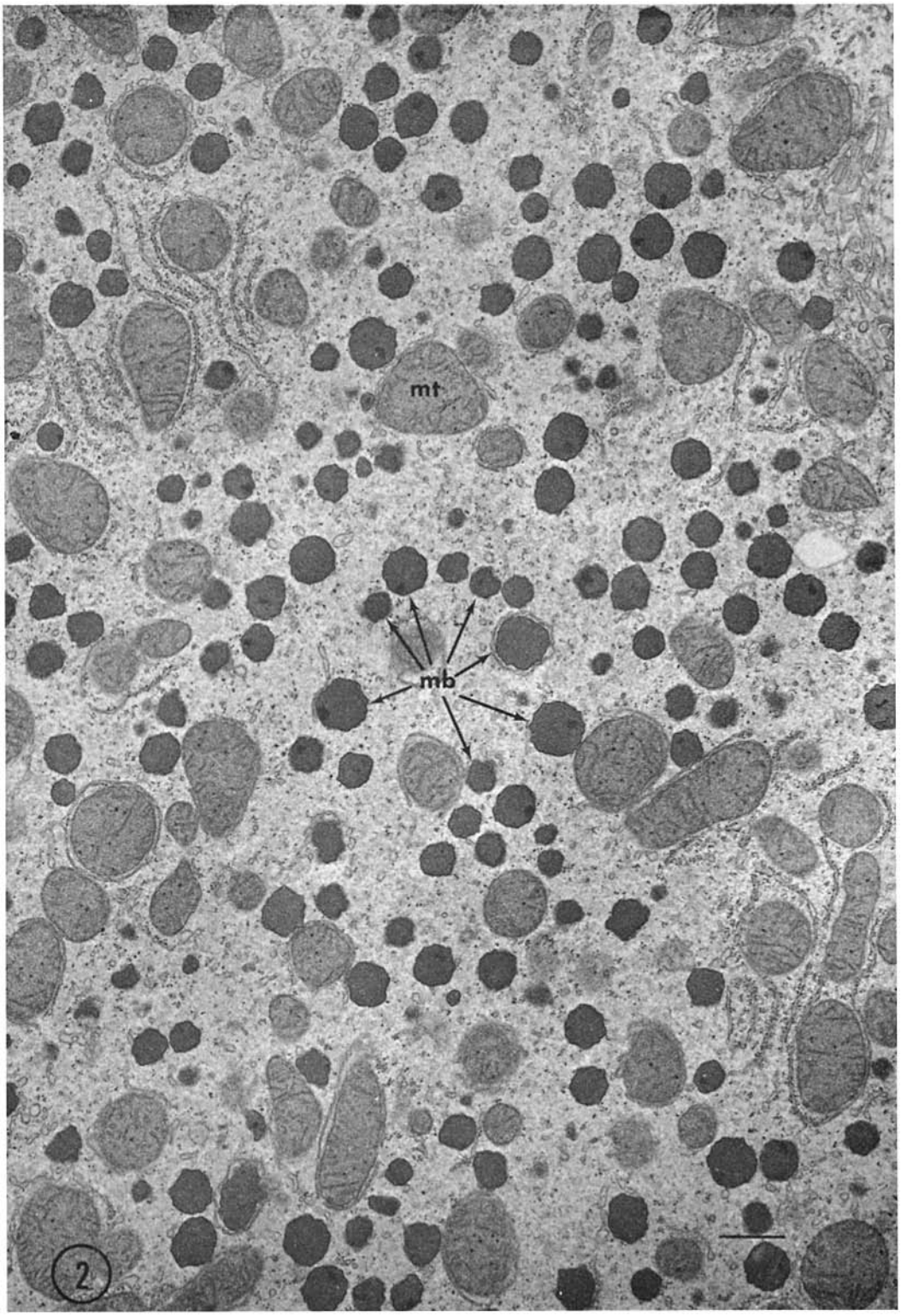

Figure 2 Intact male rat; CPIB, 4 wk. Liver cells show a marked increase in the number of microbodies (*mb*). Many microbodies lack nucleoids. *mt*, mitochondria. × 9600.

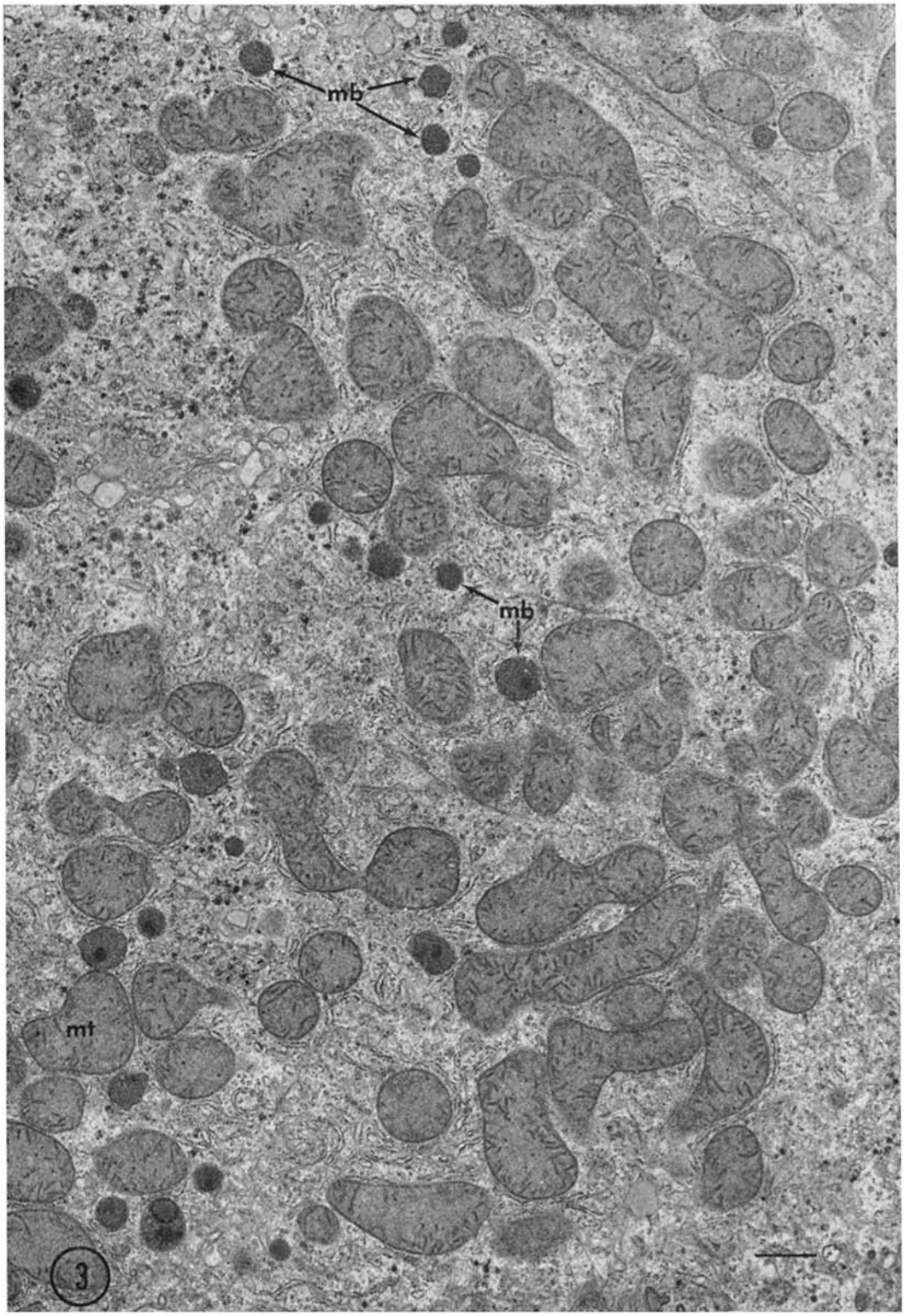

Figure 3 Castrated male rat; CPIB + estradiol benzoate, 4 wk. There is no increase in the number of microbodies (*mb*). × 9600.

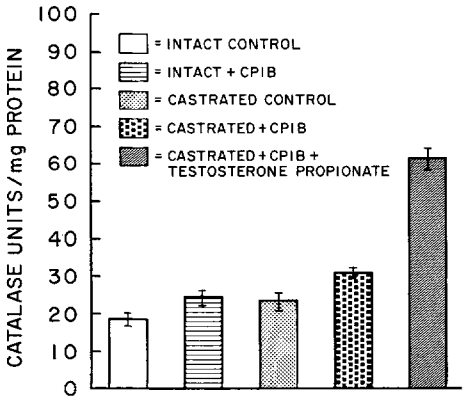

FIGURE 4 Effect of CPIB on catalase activity in intact female rats and in castrated female rats given testosterone propionate.

control values at all three intervals examined but no significant sex difference was apparent. Compared to fetuses from mothers that did not receive CPIB, fetuses whose mothers received CPIB showed an increase in the number and diameter of hepatic microbodies in both sexes. A marked and approximately equal increase in hepatic catalase activity occurred in both sexes on the day of birth, but this activity decreased by 5 days after birth (Fig. 10).

The effects of other agents which decrease binding of thyroxin to serum protein (trypan blue, novobiocin, 2,4-dichlorophenoxyacetic acid, acetylsalicylic acid) were not consistent with respect to microbody proliferation, catalase activity, and hypolipidemia. No uniform and consistent change in these three parameters occurred with the agents examined.[1]

DISCUSSION

The difficulties and sources of error in quantitative comparisons from electron micrographs alone have been described (15, 16), and de Duve and coworkers have recently discussed discrepancies between numerical assessment and protein content of microbodies (peroxisomes) (7). Nevertheless, the uniform method for assay of catalase activity used in these experiments indicates reproducible biochemical changes in catalase after gonadectomy and sex hormone supplementation. Moreover, the biochemical determinations correlate very well with the ultrastructural observations.

Gonadectomy

It is apparent that, in rats, microbody proliferation following CPIB is sensitive to sex hormones. Whether this sensitivity is inherent in the microbody as such or is related to a different microenvironment within the liver cell owing to differences in drug metabolism in the two sexes cannot be stated with certainty from these experiments.

Sex differences in the response of rodent liver to drugs has been demonstrated in a number of instances (17). For example, the microsomes from livers of male rats were more active than those from livers of female rats in *n*-demethylation of morphine (18) and in the oxidation of hexobarbital (19). The latter effect is obviated in females by the administration of testosterone (19), and starvation or adrenalectomy may diminish or abolish the sex difference in metabloism of drugs by rat liver microsomes (20, 21). In female rats, the stimulation of microsomal drug metabolism is more closely paralleled by anabolic than by androgenic effects of testosterone (22).

Gonadectomy has little effect on the lowering of total lipids and cholesterol by CPIB (23, 24). The contrast between the dependence of CPIB-induced microbody proliferation on gonadal hormones and the independence from gonadal hormones of hypolipidemia following CPIB suggest that the two responses need not be related, and experiments in thyroidectomized animals (discussed below) support this interpretation.

Sex difference in catalase activity has been reported but refers largely to the extraparticulate catalase which comprises only a small proportion of the total (25). Adams demonstrated that in mice castration or castration plus adrenalectomy resulted, in 48 hr, in a significant fall in catalase activity, although in females ovariectomy alone caused no change (26). Adams also showed that in males depression of catalase activity by castration is reversed or restored by injection of testosterone, while in females testosterone elevates their normal lower level of catalase activity to that of the male. The microbody-catalase responses in rats in the present experiments are, therefore, in general agreement with earlier reports.

Most sex differences in drug metabolism are seen only in the rat (17, 19); sex difference in

[1] Azarnoff, D. and Svoboda, D. Unpublished data.

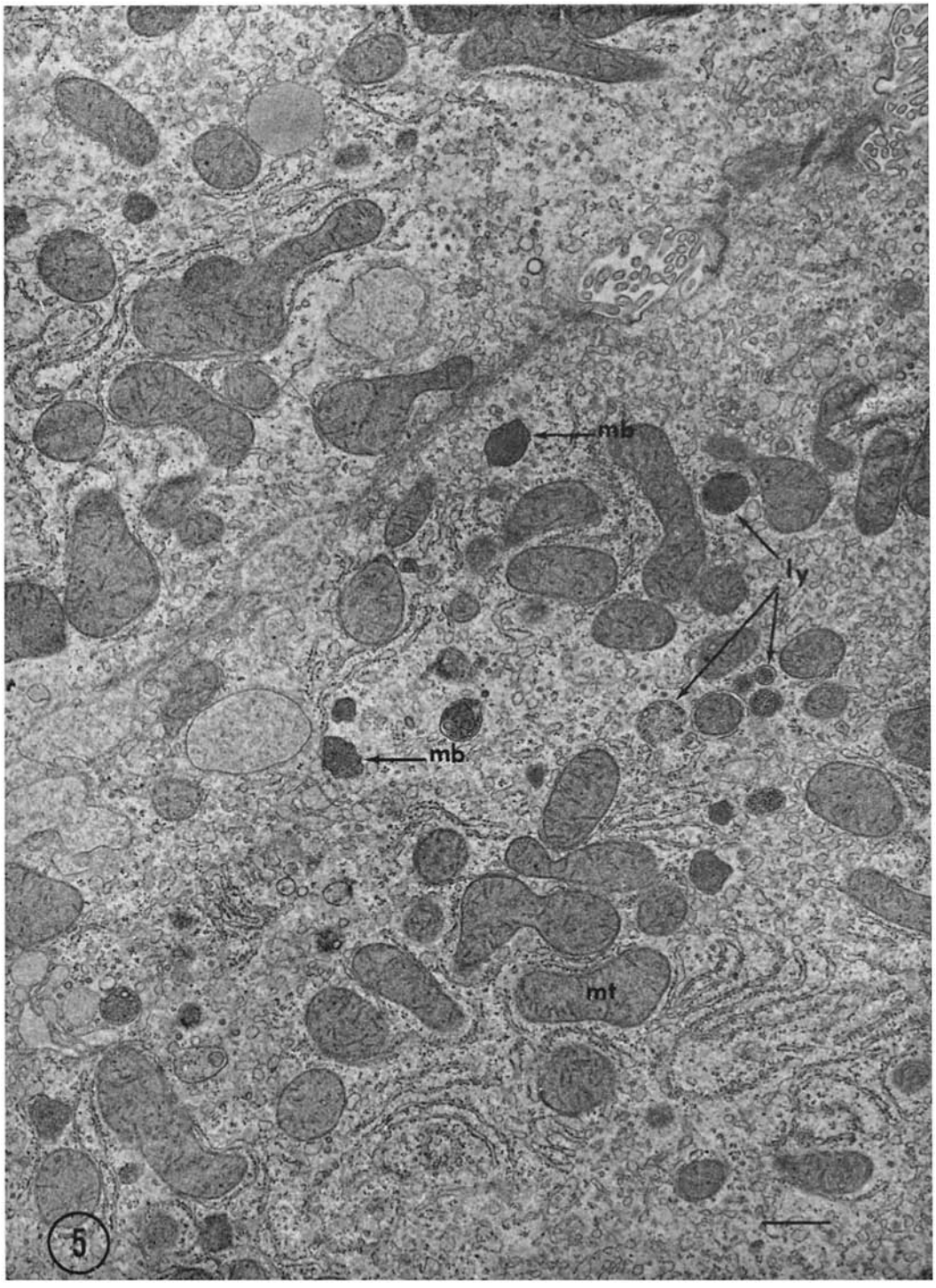

Figure 5 Intact female rat; CPIB, 4 wk. There is no increase in number of microbodies in liver cells. Instead, the cells resemble those of castrated male rats given estradiol benzoate with CPIB (compare to Fig. 3) or those of intact controls of either sex not given CPIB. *mt*, mitochondria. *ly*, lysosomes. × 9600.

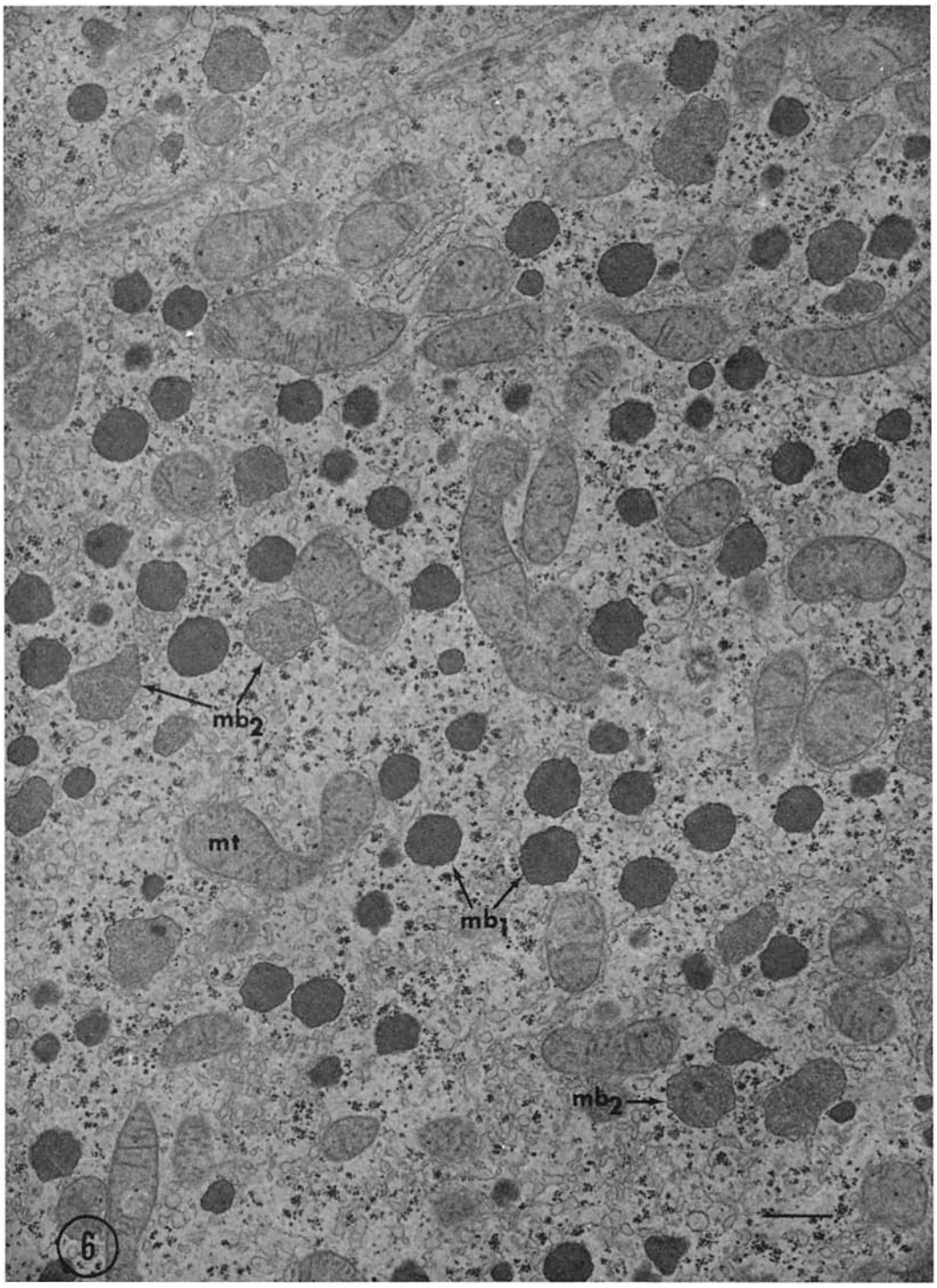

Figure 6 Castrated female rat; CPIB + testosterone propionate, 4 wk. Liver cells contain numerous microbodies and resemble the cells of intact male rats given CPIB (cf. Fig. 2). In addition to those with a typically dense matrix (mb_1), several microbodies have a pale matrix with decreased electron opacity (mb_2). Although the reason for differences in electron opacity of the matrix is unknown, similar differences are present in hepatic microbodies of intact male rats given CPIB (1). *mt*, mitochondria. × 9600.

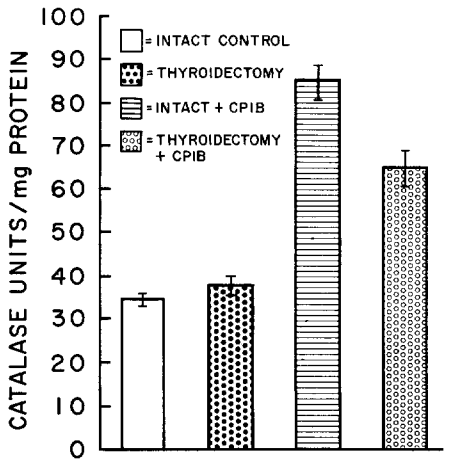

FIGURE 7 Effect of CPIB on liver catalase activity in male rats after thyroidectomy.

hypolipidemic response to CPIB in man, in clinical trials of the drug, has not been noted to any significant degree. Hess and coworkers observed, however, that female rats responded more slowly than males to CPIB-induced elevation of mitochondrial glycerol-1-phosphate dehydrogenase activity (27).

The presence of androgens *per se*, or their anabolic effects, may be important factors in determining sex variation in activity of microsomal drug-metabolizing enzymes. The extrapolation of observations of microsomal enzyme induction to microbody enzyme induction can only be tentative at the present time, but it appears that the simultaneous increase in microbodies and in the activity of their main constituent, catalase, represents a new form of enzyme induction analogous to the increase in smooth endoplasmic reticulum and concomitant increase in microsomal enzyme activity following administration of more familiar enzyme inducers such as dichlorodiphenyldichloroethane (DDD) (28) or phenobarbital (29).

It may be of interest to note that, in CPIB-treated male rats, while catalase activity is increased, there is 80% reduction in activity of D-amino and L-α-hydroxy acid oxidases in hepatic microbodies (30). This observation indicates that the main enzymes of these organelles do not respond in parallel fashion to the effects of CPIB. It appears, therefore, that, following administration of CPIB, there may be either considerable dissociation among the enzyme constituents of individual microbodies or that a heterogeneous population of these organelles arises.

Fetal Rats

Several investigators (31–33), studying fetal rat and mouse liver, have reported that the earliest appearance of microbodies occurs between the 15th and 18th day of gestation and that their number increases up to the day of birth. Tsukada and coworkers (34) described in detail the number and development of microbodies and enclosed nucleoids during postnatal development and suggested, as did Essner (31), that microbodies originated from granular endoplasmic reticulum. Similarly, Higashi and Peters (35) showed that, in microsomes, catalase was first associated with the granular fraction.

From the present experiments, it is clear that the sex difference in microbody response to CPIB observed in adult rats is not manifest in fetal and early neonatal rat liver. Previous studies have shown that, before puberty, there is no sex difference in rats with respect to their response to hexobarbital (19). Related studies in mice, guinea pigs (36), and rabbits (37) have shown that the fetal livers of these species lack several of the drug-metabolizing, microsomal enzymes that are present in the livers of adults of the respective species. Changes in microbodies and catalase activity, after the 5th postnatal day, were not studied in the present experiments, but Tsukada et al. (34) described continuous increase in catalase activity of livers from male rats up to 60 days. In considering the microbody and catalase response of rat fetal liver cells to CPIB, it is of interest to compare the present findings to related observations in other conditions in which liver cells divide rapidly. For example, Dalton suggested that the number of microbodies in liver tumors was inversely proportional to the growth rate of the tumor cells (38). But growth rate or mitotic rate may not be significant factors in determining response of microbodies to CPIB, since cells in fetal or regenerating (2) liver appear to be comparable to cells in normal adult liver in their capacity to produce microbodies. On the other hand, from additional experiments, it was evident that cells of chemically induced, but well differentiated and comparatively slowly growing primary hepatomas do not produce an increased number of microbodies in response to CPIB.[2]

[2] Svoboda, D., and D. Azarnoff. Unpublished observations.

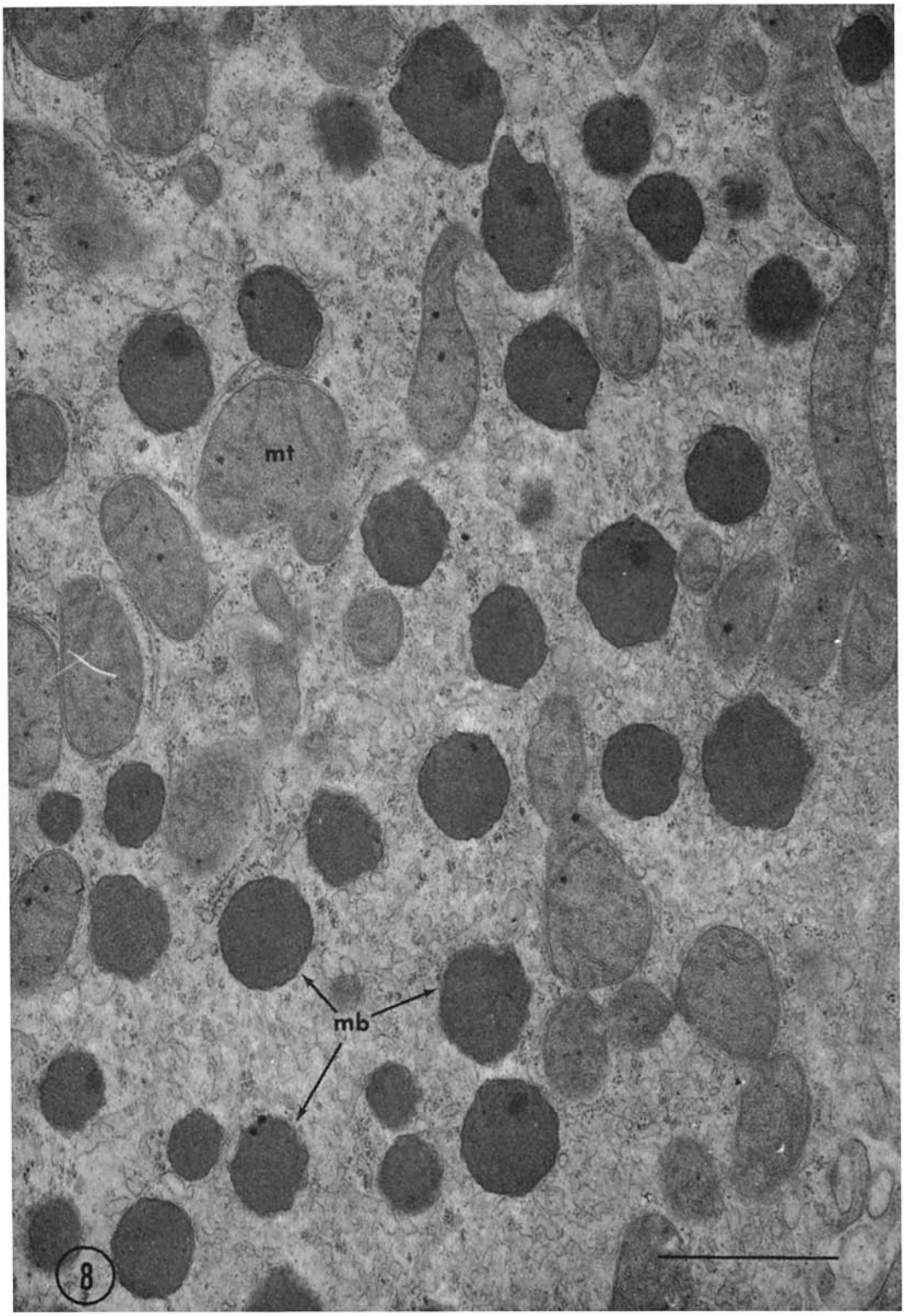

Figure 8 Male rat; thyroidectomy; CPIB, 4 wk. Liver cells show the typical increase in number of microbodies (*mb*). *mt*, mitochondria. × 28,800.

Thyroidectomy

It has been shown that endogenous thyroxine is necessary for the effect of CPIB in decreasing serum lipids (8, 11, 24). In the present experiments, the observation that, despite thyroidectomy, hepatic microbody proliferation occurs with CPIB feeding indicates that the hypolipidemic effect of CPIB is separable from the microbody-stimulating effect; and, conversely, that microbody proliferation may be independent of hypolipidemia *per se*.

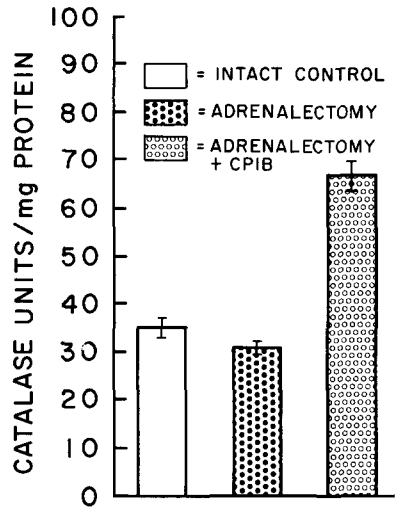

FIGURE 9 Effect of CPIB on liver catalase activity in male rats after adrenalectomy.

To explain the mode of action of CPIB on lipid metabolism, it has been suggested that the chlorophenoxyisobutyrate anion displaces serum albumin-bound acidic substrates, coenzymes, and hormones, notably thyroxine (9), with consequent uptake of thyroxine in the liver (10). It has been proposed that, because of the displacement of thyroxine by CPIB into the liver, the net result is the production of a hyperthyroid effect in the liver with relative hypothyroid levels in the rest of the body (39). However, ultrastructural changes in mitochondria from liver cells (40) and skeletal muscle cells (41) from rats given thyroxine were not apparent in any CPIB-treated animals.

Experiments with other agents that displace thyroxine from plasma protein tend to substantiate the contention that thyroxine displacement from plasma proteins by CPIB need not be the *sine qua non* of CPIB activity as far as microbody proliferation is concerned. For example, novobiocin and trypan blue, substances that displace thyroxine from its plasma protein binding sites (42), have no effect on microbody proliferation,

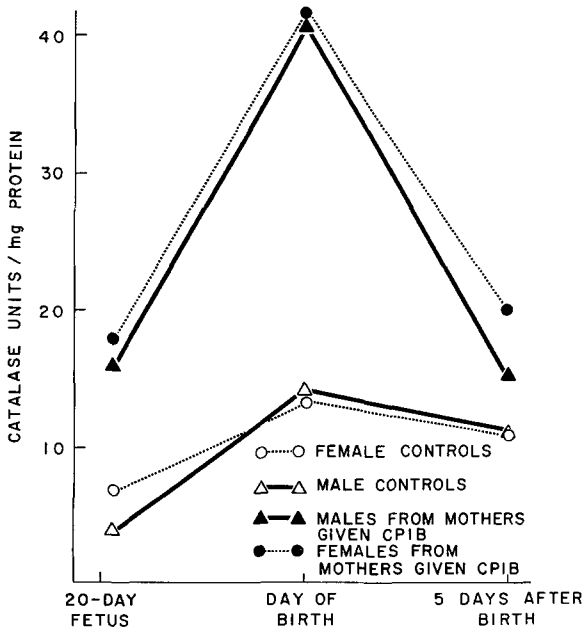

FIGURE 10 Effect of CPIB on liver catalase activity in fetal and neonatal rats of both sexes.

catalase activity, or hypolipidemia.[3] 2,4-dichlorophenoxyacetic acid, structurally similar to CPIB and capable of lowering serum cholesterol (43), does not affect microbody number or catalase activity. Perhaps acetylsalicylic acid more closely approximates CPIB in its effects on all three parameters, although its effect on the number of microbodies (44) and catalase acitivty is not so pronounced as that of CPIB.

Chang and coworkers (45) observed species differences in the degree of displacement by CPIB of thyroxine from the various fractions of plasma protein and pointed out that, in the rat, CPIB depressed only the relatively less important albumin-bound thyroxine rather than that portion of thyroxine bound to alpha-globulin. Since the importance of albumin binding of thyroxine in the rat is still open to dispute, no conclusion can be drawn regarding the relationship of thyroxine displacement to microbody proliferation in this species. In any event, it appears that the effect of CPIB on free thyroxine concentration in circulating blood is much less in the rat than in man or the dog (45), and this observation, coupled with the absence of changes in mitochondrial structure that typically occur owing to local elevation of thyroxine concentration in liver cells, suggests that thyroxine displacement is not a sufficient mechanism for microbody proliferation. The possibility exists, however, that displacement of other anionic compounds, such as androgenic sulfates, from their plasma protein-binding sites may account for the increase in number of microbodies.

Of special relevance to the present studies are the experiments of Blum and Wexler (46) who showed that even in the unicellular organism, *Tetrahymena pyriformis*, a ciliated protozoan known to contain peroxisomes (6), CPIB causes a 20% increase in catalase activity.

Adrenalectomy

Previous studies (23, 47) have shown that the effectiveness of CPIB in rats varied with seasonal fluctuations in thyroid and adrenocortical activity, and that the maximal effect of CPIB on rat serum cholesterol corresponded to the period of maximal adrenocortical and thyroid function. Best and Duncan (8) also found that adrenalectomy in rats abolished the effects of CPIB on lipids, and Thorp and Barrett (24) stated that adrenal 17-ketosteroids were necessary for lowering of lipids by CPIB. In the present experiments, adrenalectomy in male rats caused a slight decrease in catalase activity, as has been noted previously in rats (48) and mice (49, 50), but did not inhibit the CPIB-induced elevation of catalase activity (Fig. 9) or proliferation of microbodies. It is clear, therefore, that neither thyroid or adrenal hormones are necessary for CPIB-induced microbody proliferation.

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