juction with our previous studies of antioxidant modification of the acute ethanol-induced hepatic lesion, further accents the possible role of lipid peroxidation as a determinant in cell injury and the role of antioxidants as liver protective agents.

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Observations on the Inhibitory Influence of Plethora on Erythropoietic Action of Androgens in Hypophysectomized Rats* (32671)

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The present studies were prompted by two observations made in this laboratory: (a) that the erythropoietic response to norethandrolone, as measured by ^{59}Fe uptake and reticulocyte counts, was abolished by blood transfusions in hypophysectomized rats, and (b) whereas the hypophysectomized rat, 2 weeks after surgery, responds readily to norethandrolone, no response to equal doses of testosterone propionate was obtained (1). Further elucidation of the relationship between the plethoric state and androgen activity seems important to an ultimate understanding of the mechanisms by which androgens stimulate erythropoiesis.

The literature concerning the action of androgens on erythropoiesis has become extensive and many suggestions have been made on the mechanism of their stimulatory effect. In general, the following points of view are still in consideration: (a) androgens act by stimulating erythropoietin production (2–4) or they alter the sensitivity of the kidney in such a way that there is increased ESF production (3); (b) androgens act in some way other than by stimulating ESF production, such as an interaction in some manner with ESF (1,5) or with the stem cell so that stimulation occurs without requiring increased amounts of ESF (6); or (c) that androgens interfere or interact in some way with an inhibitor substance (7).

The use of the polycythemic or plethoric animal has been intimately involved in all the studies from which these various ideas have been formulated, and the plethora has influenced the response to androgens in a variety of ways. Gurney *et al.* (8–11) found, in the mouse, that various degrees of plethora would reduce, but not eliminate, the response

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to testosterone; the inhibition varied directly with the degree of plethora induced. Gordon *et al.* (12) demonstrated, in normal testosterone treated rats, that both the amount of erythropoietin (ESF) in the plasma and the formation of renal erythropoietic factor (REF) by the kidney were diminished by a transfusion. In addition, some evidence has been introduced which suggests that the manner in which the plethora is produced plays a role in the results obtained; for example, Nates and Wittek (5) found that the erythropoietic response to testosterone was eliminated in mice in which the polycythemia was induced by a transfusion, but if the polycythemia was produced by hypoxia, the mice responded to the androgen. Jepson and Lowenstein (6) also used hypoxia to induce a polycythemia and their mice also responded to androgen treatment.

With the variety of findings presented in the above studies in mind, namely that the plethora decreases the amount of circulating ESF, that the manner of induction of the plethora is involved, and that the degree of the plethora is an important factor, the present experiments were performed to determine whether or not these factors play a role in our observations on androgen inhibition in hypophysectomized rats.

Materials and Methods. Female Holtzman rats 2–3 months of age were used. Rats were given food and water *ad libitum*, supplemented once a week with lettuce. In experiments 1 and 2, rats were used 2 weeks after hypophysectomy, and in experiment 3, approximately 1 year after surgery. The androgens used were either norethandralone[2] or testosterone propionate diluted in sesame oil and injected subcutaneously. The erythropoietin[3] used was human urinary erythropoietin diluted 1 unit/ml in saline and injected subcutaneously as 0.5 unit twice a day. When plethora was induced by transfusion, oxylated blood was obtained from normal donor rats, centrifuged, the supernatant drawn off, and reconstituted to a hematocrit of approximately 70%, and each rat transfused with an equivalent of 3.5 ml (hematocrit 70%) of this concentrated blood via tail vein. In all cases the transfusions were given 2 days prior to the start of the treatment. Plethora by means of hypoxia was accomplished by exposure to a simulated altitude of 23,000 feet in a cylindrical chamber with a capacity of 250 liters and evacuated so that the rate of airflow through the chamber was 32 liters/min; the exposure was for 4 hours/day for 10 days starting 2 days after surgery and ending 2 days before treatment was started. In all experiments ^{59}Fe incorporation was measured by calculating the percentage of uptake 18 hours after a tail vein injection of 1 μC diluted in 1 ml of saline. The injection was done at approximately 4:00 p.m. on the day before autopsy. In the calculation the total blood volume was estimated to be 5.23% of body weight for those rats having received either transfusions or hypoxic exposure, and 4.87% for all others (13). Reticulocyte percentages were based on 1000 cells counted on smears stained with new methylene blue. Hematocrits were done by the microhematocrit method.

Procedure and results. Experiment no. 1. The importance of circulating ESF was investigated as follows. All rats were hypophysectomized, and injections were started 14 days after surgery. If a transfusion was given, this occurred 2 days before the injections were started. The rats were divided into 6 groups and treated as follows: (i) daily injections of sesame oil for 5 days; (ii) daily injections of 1.0 mg of norethandrolone for 5 days; (iii) transfusion followed by 5 consecutive daily injections of sesame oil; (iv) transfusion as above but 1.0 mg of norethandrolone was substituted for the sesame oil; (v) transfusion followed by daily injections of 1 unit of ESF for 5 days; and (vi) transfusion followed by daily injections of 1.0 mg of norethandrolone and 1 unit of ESF for 5 days.

The results are presented in Table I. The norethandrolone in hypophysectomized rats induced the usual increase in ^{59}Fe uptake and reticulocyte level, and the transfusion abolished this stimulatory effect. The ESF treatment in the transfused–hypophysecto-

[2] Nilevar, Searle Company.

[3] Obtained from the Erythropoietin Subcommittee, N.I.H.

TABLE I. Effects of Norethandrolone and ESF on ^{59}Fe Uptake and Reticulocyte Level in Hypophysectomized Adult Female Rats.[a]

Treatment	No. of rats	Body wt. (gm)	Hematocrit (%)	^{59}Fe uptake (%)	Reticulocytes (%)
Sesame oil only	15	182 ± 2.20	40 ± 0.53	11.3 ± 1.06	0.5 ± 0.15
Norethandrolone (1.0 mg daily)	11	183 ± 3.53	41 ± 0.72	25.2 ± 2.27	2.1 ± 0.44
Transfused and sesame oil	10	208 ± 1.60	49 ± 0.48	5.5 ± 0.39	0.5 ± 0.07
Transfused and norethandrolone	9	217 ± 3.62	48 ± 0.87	5.8 ± 0.80	0.5 ± 0.10
Transfused and ESF (1 unit daily)	10	200 ± 2.26	50 ± 0.76	16.2 ± 0.56	1.2 ± 0.08
Transfused, norethandrolone, and ESF	11	210 ± 1.69	50 ± 0.47	17.2 ± 0.50	1.3 ± 0.09

[a] Values are ± SE.

TABLE II. Effects of Norethandrolone and Plethora on ^{59}Fe Uptake and Reticulocyte Level in Hypophysectomized Adult Female Rats.[a]

Treatment	No. of rats	Body wt. (gm)	Hematocrit (%)	^{59}Fe uptake (%)	Reticulocytes (%)
Sesame oil only	7	192 ± 2.33	39 ± 0.60	15.3 ± 1.85	0.6 ± 0.09
Norethandrolone (1.0 mg daily)	9	198 ± 2.66	40 ± 1.10	32.6 ± 3.76	2.6 ± 0.50
Transfused and sesame oil	7	213 ± 2.81	51 ± 1.17	6.6 ± 0.61	0.1 ± 0.03
Transfused and norethandrolone	10	218 ± 2.44	49 ± 0.46	6.6 ± 0.65	0.2 ± 0.05
Hypoxia and sesame oil	6	214 ± 2.85	50 ± 1.30	10.9 ± 0.77	0.3 ± 0.05
Hypoxia and norethandrolone	10	216 ± 3.68	49 ± 0.58	11.2 ± 1.23	0.3 ± 0.07

[a] Values are ± SE.

mized rats induced a threefold increase in both ^{59}Fe uptake and in reticulocyte percentage. When norethandrolone was combined with the ESF in transfused hypophysectomized rats there was no difference in ^{59}Fe uptake or reticulocyte level from the animals receiving the ESF only. Provision of exogenous ESF did not restore the effectiveness of the androgen. This indicates that it is not the absence of ESF that is responsible for the inhibitory influence of the plethoric state. It suggests further that the mechanism of androgen action is not one of interaction with ESF as has been suggested.

Experiment no. 2. To test the possible influence of the manner in which the plethora is induced, rats hypophysectomized 2 weeks previously were grouped and treated as follows: (i) daily injections of sesame oil for 5 days; (ii) daily injections of 1.0 mg of norethandrolone for 5 days; (iii) transfusion followed by 5 consecutive daily injections of sesame oil; (iv) transfusion as above but 1.0 mg of norethandrolone was substituted for the sesame oil; (v) exposure to hypoxia followed by 5 daily injections of sesame oil; and (vi) exposure to hypoxia as above but 1.0 mg of norethandrolone was substituted for the sesame oil. The results are shown in Table II. It can be seen that the response to the androgen was abolished by plethora, whether it was induced by transfusion or by hypoxia. It might be noted that, although the reaction to the androgen was in the same direction, the transfused rats exhibited a lower ^{59}Fe incorporation than did the rats exposed to hypoxia, even though the hematocrits were the same. Possibly this is due to the acute nature of the transfusion and/or some adaptation to the intermittant hypoxia. In any event, the results show clearly that the inhibition of the erythropoietic response was due to the existence of the plethoric state and not to any factor concerned with the manner in which it was produced.

Experiment no. 3. To test whether the

TABLE III. Effects of Testosterone Propionate on ^{59}Fe Uptake and Reticulocyte Level in Hypophysectomized Adult Female Rats, Approximately 1 Year After Surgery.[a]

Treatment	No. of rats	Body wt. (gm)	Hematocrit (%)	^{59}Fe uptake (%)	Reticulocytes (%)
Sesame oil only	5	206 ± 4.8	37 ± 0.50	30.6 ± 2.80	1.0 ± 0.10
Testosterone propionate (3 mg daily)	8	208 ± 6.3	40 ± 0.74	52.9 ± 3.01	3.2 ± 0.30

[a] Values are ± SE.

degree of plethora was responsible for our findings in hypophysectomized animals, rats which had been hypophysectomized approximately 1 year before treatment were studied. These animals, in contrast to those used above (in a state of relative polycythemia), had reestablished an erythropoietic balance in keeping with their metabolic demands. The rats were divided into 2 groups: (a) a sesame oil treated control group and (b) an experimental group treated with 3 mg of testosterone propionate per day for 5 days. The dose selected corresponds to that used in our previous study (1). The results are shown in Table III. The ^{59}Fe uptakes for the control animals were at a level characteristic of those in preoperated normal animals, or even a little higher; they were much higher than the depressed condition seen at the 2-week interval in the other two experiments. Nevertheless, the testosterone produced a significant increase in the ^{59}Fe uptake and reticulocyte level. This is in direct contrast to our finding at 2 weeks after surgery when the testosterone was ineffectual.

Discussion. Many reports in the literature confirm the depressing influence of plethora on androgen activity. However, there are many discrepancies concerning the severity of the suppression. It is difficult to compare the various reports since dosage of androgen, type of androgen, amount of blood transfused, method of inducing the polycythemia, and the timing of the injections all vary, and often the final hematocrits are not reported. In general, complete suppression has been reported in mice (5) and in hypophysectomized rats (1) made polycythemic by transfusion, with several other reports on transfused mice and rats (2,3,8–11) indicating varying degrees of suppression.

Assuming that circulating ESF is reduced in the plethoric animal, Nates and Wittek (5) and we (1) explained the lack of erythropoietic effect of the androgens in such polycythemic animals as due to this lack of ESF; in other words, the presence of ESF is necessary for androgen action. The work of Schooley (4), when testosterone propionate injections were combined with simultaneous injections of an antierythropoietin immune serum and the erythropoietic action of the androgen was abolished, also might be interpreted as indicating that the presence of ESF is necessary for androgen activity. The first experiment in the present report indicates that the reduction or absence of ESF is not the explanation for the inhibitory effect of plethora and also suggests that an interaction of ESF and androgen is not the mechanism by which androgens stimulate erythropoiesis. Nates and Wittek (5) also showed that animals made polycythemic by a transfusion would not respond to androgen injections, while animals made polycythemic by means of hypoxia would respond. The results of our second experiment indicated no difference in response whether the plethora was induced by transfusion or by hypoxia.

Therefore, the results of the first two experiments indicate that the suppression of the erythropoietic response to androgens by the plethoric state is not due to a lack of ESF with which the injected androgen may react, nor is it due to any peculiarity about the manner in which the plethora is produced. It should be noted that in the first two experiments complete suppression occurred in the presence of relatively low hematocrits. This is thought to have a

bearing on the finding in the third experiment. At 2 weeks after hypophysectomy, the rat is a relatively polycythemic animal and the red cell mass is relatively much higher with respect to the animal's needs than it is for the normal rat. Since the older hypophysectomized, erythropoietically-balanced rats responded, one might surmise that it was the degree of the relative plethora existing in the hypophysectomized rats 2 weeks after surgery that was responsible for the failure to see a response to testosterone. It should also be noted that this work suggests that there is a difference in the potency of the two hormones involved in the ease with which erythropoietic action is suppressed. This is in accord with the findings of Luis Sanchez-Medal and co-workers, (personal communication) that certain anabolic steroids are more active erythropoietically than is testosterone.

From these experiments it seems that the degree of plethora would explain our findings. However, in treating normal animals in a manner similar to the hypophysectomized animals, we have never seen complete suppression of the norethandrolone activity. Also, Gordon *et al.* did not find complete suppression of ESF and REF in transfused normal rats having hematocrits of 65% to 70%. This latter observation is of interest because hematocrits at this level would be comparable to estimated levels for the hypophysectomized rats 2 weeks after surgery if one took into account the degree of the relative polycythemia [estimated at 22% (16)] and added it to the amount of increase due to the transfusion.

Since the mechanism by which androgens produce an erythropoietic effect has not been entirely resolved, the means whereby the plethora inhibits this effect must accordingly remain unsolved. Several investigators have proposed that testosterone acts by stimulating ESF production (2,3). Gordon *et al.* (12) reported that plethora reduced the production of REF by the kidney and suggested this as an explanation for the reduced levels of ESF in transfused, testosterone treated rats. The relationship of REF to the plethoric state, however, is not exactly known. It has also been reported that plethora induces a production of an inhibitor of erythropoiesis (14,15); on the basis of clinical evidence, Stohlman (7) suggested that testosterone might interfere with the production or action of this inhibitor, thereby permitting either an increased effectiveness of ESF, or a detection of the normal action of ESF, if the inhibitor was present in excess. Also, Gordon *et al.* (12) pointed out the possibility of a relationship between this inhibitor and suppression of REF. The possibility of plethora inducing a production of inhibitors to such a degree that they counteract the effect of the androgen warrants further investigation.

Further extensions of the present studies are being conducted to incorporate the possibility of an inhibitor being involved, and to test whether complete inhibition, according to our methods, can ultimately be obtained in normal rats.

The present experiments indicate that the erythropoietic response to androgens is dependent on the degree of plethora, and that it is not involved with the manner in which the plethora is induced or with the fact that ESF is reduced in such polycythemic animals.

Summary. Experiments on plethoric, androgen treated, hypophysectomized rats indicate: (a) the inhibitory effect of plethora on the erythropoietic activity of androgens is not due to a lack of ESF; (b) the manner in which the plethoric state is obtained is not a factor in the inhibitory effect; and (c) by indirect evidence, that the failure to see an erythropoietic response to testosterone propionate in hypophysectomized rats 2 weeks after surgery is related to the relative degree of plethora existing in this animal.

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Evidence for Transplacental Passage of the Natural Carcinogen Cycasin and Its Aglycone (32672)

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It was recently reported from this Laboratory that rats exposed *in utero* to cycasin, methylazoxymethyl-β-D-glucoside, developed tumors in later life (1) and that malformations of the central nervous system and extremities could be induced in fetuses when the aglycone of cycasin, methylazoxymethanol (MAM) was administered intravenously to pregnant hamsters on the eighth day of gestation (2).

These findings suggested but did not conclusively prove a transplacental passage of the compounds. Although the pattern of urinary and fecal excretion of cycasin was found comparable in pregnant and nonpregnant female rats (1), the possibility remained that small amounts of cycasin or MAM might be stored in the mammary glands and excreted with the milk postpartum. Such an event could have provided the mechanism by which the tumors were induced in view of the observations of Mugera *et al.* (3) who suggested that the toxic material was excreted with the milk when lactating animals were fed cycad materials. Similarly, the induced malformations in the fetuses of hamsters receiving the highly toxic MAM could conceivably have been the result of maternal injury without cycasin or MAM directly being involved.

To clarify the route of cycasin effect in fetuses, experiments were designed to (i) search for evidence of storage of cycasin or MAM in lactating mammary glands of rats fed crude cycad material during pregnancy, (ii) determine whether transplacental passage of cycasin or MAM occurred in pregnant rats and hamsters, and (iii) establish the presence of cycasin or MAM in secreting mammary glands when cycasin is administered to lactating rats.

Material and Methods. Experimental animals. Forty-seven female Fischer rats, 12 of which served as controls, and 10 female golden hamsters, including 3 controls, were used. The rats were obtained from the NIH animal breeding facilities and the hamsters from a commercial hamster colony in Newfield, New Jersey. Females of both species were mated in our laboratory and the day on which spermatozoa were observed in vaginal smears was designated as day 1 of pregnancy. The following groups of animals were used:

Group I. Ten pregnant rats were fed commercially available ground Purina chicken feed diet. Crude cycad meal containing 3% cycasin was added to the diet which was fed