TABLE I. Standard HCl vs Dioxane, Dioxane-HCl and Glucuronidase.

Urine batch	mg Ks/l urine: (a) Standard HCl total	(a) Dioxane total	α	β	(b) Glucuronidase total	a + b	Dioxane-HCl: Total	α	β
NMU 24	10.2	2.5	1.3	1.2	8.2	10.7	12.0	10.3	.94
27	9.2	3.8	1.5	2.0	7.4	11.2	10.4	8.4	1.9
124	9.2	3.8	1.4	2.3	5.8	9.6	9.5	7.1	2.4
153	10.2	4.2	2.2	1.7	6.8	11.0	11.7	10.2	1.8
168	10.7	4.6	2.2	2.4	7.2	11.6	12.2	9.9	2.2
215A	11.0	4.4	2.1	2.2	7.4	11.8	13.5	9.8	3.0
NMU 216	9.8	4.4	1.3	2.8	7.5	11.9	13.3	10.7	2.4
LPU 20	5.5	2.2	1.6	.6	3.4	5.6	6.1	5.1	.7

effects maximum hydrolysis was found by dissolving the residue in 10 cc of 1,4 dioxane; after adding 0.1-1.4 cc (1-14%) concentrated HCl, the preparation was heated in a steam bath for 10 minutes (see Fig. 1). It can be seen that 8-10% HCl gives a maximum hydrolysis of the urinary ketosteroids. Results of the dioxane-HCl hydrolysis on various urine residues are shown in Table I. It can be seen from these data that about 17% more steroids are formed than by the standard HCl procedure. About 20% of the total steroids were found to be of beta type. Heating with an excess of acid or heating for a long period of time results in the destruction of the beta steroids. It is important to point out that longer heating causes destruction of the ketosteroids. Thus, Urine NMU 153 heated with dioxane HCl for 10 minutes resulted in the release of 2.1 mg of beta steroids. After heating for 15 minutes, the beta steroids fell to 1.97 mg, and after heating for one hour the beta steroids were 0.94 mg.

Summary. The total ketosteroids of urine may be readily hydrolyzed by dioxane-hydrochloric acid. This hydrolysate contains about 20% of the total steroids as beta type.

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Increased Metabolic Rate without Thyroid Participation on Injection of Rats with Pituitary Erythropoietic Fractions.* (21216)

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As reported recently, extracts from sheep pituitary can be obtained(1) containing an erythropoietic principle which repairs the anemia resulting from hypophysectomy(1-3), prevents the development of the neonatal anemia(4,5), and produces polycythemia in the normal(6), hypophysectomized(7,8), and adrenalectomized rat(2). Reticulocytes and normoblasts appear in the peripheral blood of hypophysectomized rats injected with this erythropoietic factor, and their bone marrow is stimulated. Inasmuch as reports have appeared in the literature indicating that increased activity of the bone marrow is accompanied by an elevated metabolic rate(9-11), the caloric output of rats injected with the pituitary erythropoietic fraction was examined.

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Methods. Female rats of the Long-Evans

strain were hypophysectomized when 26-28 days old and were maintained for 45 days.† During this postoperative period the red cell volume decreased to a level half that of normal(6). Injections of pituitary erythropoietic preparations(1) were subsequently given daily for a period of 14 days. The substances were administered subcutaneously in a beeswax and oil mixture. The red cell volumes were determined at the end of the experimental period, using the Fe^{59} labeled cell method(12), in order to ascertain the effectiveness of these erythropoietic fractions in repair of the post-hypophysectomy anemia. Histological examinations of the target organs of the pituitary were made in order to determine the presence in the injected material of contamination by known pituitary hormones. Completeness of hypophysectomy was confirmed at autopsy by examination of the sella turcica, and no data have been included in the tables except from animals completely hypophysectomized. The basal metabolic rate was determined two days before the termination of the experiment. A fast of 10 hours preceded this test.‡

Results. All of the pituitary preparations listed in Table I stimulated erythropoiesis and repaired the post-hypophysectomy anemia. Increased calorigenesis also characterized all groups. The caloric output increased 78 to 101% so that the metabolic rate returned from the hypophysectomized level to the normal. As the pituitary preparations which stimulated erythropoiesis also increased calorigenesis, the possibility of thyrotrophic hormone contamination in the preparations was considered. Histological examination of the thyroids showed evidence of minimal activity in thyroids of some groups, but no stimulation in others (Table I).§ The increased metabolic rate was of the same order in all groups, despite variability in thyroid histology.

The functional capacity of thyroids of the rats treated with the pituitary erythropoietic factor, as judged by their ability to concentrate tracer doses of I^{131}, paralleled these histological findings.

In order to apply this test of function, red cell volume determinations were first made by withdrawal of blood from the external jugular. A small dose of I^{131} (1 μc) was then injected intraperitoneally and the thyroids were removed 24 hours later, fixed in Bouin's fluid, and the radioactivity was counted directly in a scintillation counter(14).

The I^{131} uptake of the thyroids in groups showing histological evidence of thyroid stimulation was 2.1%, in contrast to 0.9% in the hypophysectomized controls. This increase in I^{131} uptake was, however, only a fraction of

† All rats used were injected intraperitoneally for 2 consecutive days after hypophysectomy with 0.5 cc of 0.5% Terramycin in 5% glucose. The injections were repeated once every 10 days for the period of the experiments. Rats with respiratory infections were excluded from the experimental groups. The rats were maintained on a standard laboratory diet (Diet XIV) given *ad libitum* and kept in a constant temperature room throughout the experiment (75 $\pm$ 2°F). The standard laboratory diet, given dry, was supplemented every afternoon by a wet mash of Diet I. Lettuce was given 2 to 3 times weekly. Diet XIV consists of 68.5% wheat, 5% casein, 10% fish meal, 10% alfalfa leaf meal, 5% fish oil, 1.5% NaCl, KI added (analysis 1 μg iodine per g diet). Diet I consists of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1.0% fish oil, 0.75% NaCl, 1.5% $CaCo_3$, KI added (analysis 1 μg iodine per g diet).

‡ Determination of oxygen consumption was performed by the method and with the apparatus described by Kleiber(13). Surface area was calculated by the formula: $m^2 = \frac{9\sqrt[3]{(BW)^2}}{10{,}000}$. We wish to thank Dr. Arthur H. Smith, University of California, Davis, for his cooperation in confirming our determinations of oxygen consumption.

§ Thyroid activity is designated in the tables as follows: + Definite, though low, stimulation, the epithelium is low and the cytoplasm scanty; nuclei are slightly rounded and chromatin particles are more easily distinguishable than in hypophysectomized controls, and the hematoxylin stain is somewhat paler: the colloid stains a lighter eosin tint.

++ The epithelium is low cuboidal; nuclei are vesicular and light staining; there are some vacuoles in the colloid.

+++ The thyroid vesicles are smaller and epithelium is higher cuboidal; there is marked vacuolation of the colloid.

++++ Almost complete resorption of colloid has occurred.

TABLE I. Increased Calorigenesis in Hypophysectomized Rats during Administration of Pituitary Erythropoietic Fractions.

Preparations inj.	Dose, mg	No. of rats	Metabolic rate — Cal/m²/hr	Metabolic rate — % of normal	Red cell vol — ml/100 g BW	Red cell vol — % of normal	Thyroid† histology	% I^{131} uptake
II-H-114 (B)	.10	8	48.0 ± 1.7*	109	2.65 ± .10	115	+(3), ++(5)	2.1 ± .2
122 (B)	.05	7	42.6 ± 1.3	97	2.67 ± .11	116	+(6), — (2)	2.0 ± .2
112 (2)	.05	7	44.3 ± 3.0	100	2.51 ± .02	109	+(3), — (5)	1.0 ± .1
107	.10	8	45.9 ± 1.8	104	2.49 ± .11	108	+(2), — (6)	.9 ± .1
$\bar{H}$ controls	—	45	23.9 ± 1.4	54	1.50 ± .05	65	—	.9 ± .1
N "	—	15	44.0 ± 1.0	100	2.30 ± .05	100	—	7.5 ± .3

† Degree of thyroid stimulation indicated is defined in text footnote. No. of rats is given in parentheses.

* $SE = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

the normal uptake (7.5%). The I^{131} uptake was not increased in any thyroids which did not subsequently show histological evidence of stimulation.

The small contamination of thyrotrophic hormone present in some of the erythropoietic preparations¶ did not adequately explain the increased calorigenesis. However, since some degree of thyroid stimulation was caused by erythropoietically active preparations, a comparison was made between the calorigenic action of these preparations and that of the most potent thyrotrophic preparations available.‖ Groups of hypophysectomized rats were injected intraperitoneally, daily for 14 days, with doses of thyrotrophic hormone in saline ranging from 5 to 450 μg. The metabolic rates were determined after the injection had been given for 12 days. Red cell volume determinations were made at the end of the 14-day period, the blood for the determination being withdrawn from the external jugular without sacrificing the rats. The ability of the thyroid to concentrate I^{131} was determined by injecting a tracer dose of I^{131} (1 μc) immediately after determination of the red cell volume. Twenty-four hours after radio-iodine administration the rats were sacrificed, the thyroids were fixed in Bouin's fluid, and the radioactivity was counted in a scintillation counter. The histology of the thyroids was later examined. Metabolic rates and red cell volumes were likewise determined for intact rats of the same age, in order to have normal values for comparison.

No significant erythropoietic response occurred in groups receiving the lower levels (5 to 150 μg) of thyrotrophic hormone (Table II). At the higher levels (300 to 450 μg), there was only a 30% increase in red cell volume. This increase is the same as that which resulted from injection of 2.0 to 3.5 μg daily of thyroxine(6), a dose judged to be physiological in that it had been found adequate for normal growth and skeletal differentiation in the hypophysectomized rat(15).

The calorigenic output of the hypophysectomized rats (Table II) was not brought back to normal levels by even the highest doses of thyrotrophic hormone given, doses which were adequate to cause an increase in the height of epithelial cells, increased size of nuclei, almost complete colloid resorption and almost normal I^{131} uptake. Thus, thyrotrophic hormone, at doses more than adequate to repair thyroid morphology and reinstate normal I^{131} uptake, failed to increase the caloric output to the normal level. This result supported the concept that the metabolic activity of erythropoietically active pituitary preparations did not stem from their content of thyrotrophic hormone.

Increased calorigenesis resulting from injec-

¶ None of the preparations contained recognizable amounts of FSH, ICSH or growth hormone at the doses injected over the 14-day period.

‖ The purified thyrotrophic hormone used had been fractionated from beef anterior pituitary and its potency standardized in hypophysectomized rats by the histological response of the thyroids and by I^{131} uptake. By both of these criteria the preparation was effective at the minimal dose of 5-10 μg(15).

TABLE II. Calorigenic Response of Hypophysectomized Rats to Graded Doses of Pituitary Thyrotrophic Hormone.

Group	Dose of TSH, mg	No. of rats	Metabolic rate: Cal/m²/hr	Metabolic rate: % of normal	Red cell vol: ml/100 g BW	Red cell vol: % of normal	Thyroid histology	% I^{131} uptake
H inj.	.005	6	22.1 ± .8*	50	1.52 ± .05	66	±	—
	.010	5	25.1 ± .9	57	1.51 ± .06	66	+	—
	.050	6	27.0 ± 1.3	61	1.59 ± .04	69	+(++)	—
	.150	6	27.4 ± .9	62	1.73 ± .08	75	+++	4.5 ± .5
	.300	6	29.0 ± .8	66	1.96 ± .10	85	++++	6.0 ± .8
	.450	6	30.5 ± 1.2	69	1.95 ± .10	85	++++	6.9 ± .5
H controls	—	45	23.9 ± 1.4	54	1.50 ± .05	65	—	.9 ± .1
N "	—	15	44.0 ± 1.0	100	2.30 ± .05	100	—	7.5 ± .3

* $SE = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

tion of the erythropoietic factor in the absence of the thyroids would furnish conclusive evidence that the change in oxygen consumptions did not mediate through the thyroid. In order to test this point the thyroid tissue was destroyed by the administration of I^{131}. A dose of 1 mc was given at 28 days of age and followed 15 days later by a second dose of 500 μc. After a lapse of 4 months, those animals were selected in which destruction of the thyroids was most probably complete. The choice was made on the basis of poor muscle tone, persistence of juvenile hair, body growth stasis, and low metabolic rate. From a group of 30 rats, 16 were selected in which the oxygen consumption was 40% or more below normal. Half of this group was injected for 14 days daily with 100 μg of an erythropoietically active pituitary preparation (II-H-109-3). Red cell volumes and metabolic rates were determined at 2 and also at 3 weeks after the onset of injection. The remainder of the rats, in which it was judged thyroid destruction was complete, were retained as controls and red cell volumes were determined at the same intervals as in the injected animals.

The completeness of thyroid aplasia in the rats chosen for the experiment was further checked terminally by the administration of a tracer dose of I^{131} (1 μc) 24 hours before autopsy. The thyroid region was dissected, placed in Bouin's fluid in a vial, and the I^{131} uptake in the thyroid region was counted directly in a scintillation counter(6,14). Only those animals judged completely free of functional thyroid tissue, by inability to concentrate radio-iodine, were included in presenting the results in Table III.

A progressive increase in the metabolic rate occurred in the injected thyroidectomized animals during the 3-week period of injections of the pituitary erythropoietic factor (Table III). After 21 days, the increase was 36% and the metabolic rate was 87% of normal. Uninjected controls showed no change in metabolic rate during this period. The erythropoietic preparations, therefore, caused a significant increase in the metabolic rate in the absence of functional thyroid tissue. The red cell volume of the thyroidectomized rats was increased to the normal level by the dose of erythropoietic principle administered. The increase in metaboilc rate of hypophysectomized rats injected with pituitary erythropoietic fractions without thyroid stimulation is difficult to interpret. It would appear that the increase of calorigenesis is not mediated through the thyroid as increased caloric output occurred in the hypophysectomized rat without stimulation of the thyroid gland, and also in the absence of functional thyroid tissue. Furthermore, increased thyroid activity, judged by morphology and iodine uptake, occasioned by large doses of thyrotrophic hormone, was accompanied by only a small increase in metabolic rate. As the erythropoietically active hormone has not been prepared in pure form, it cannot be said at present whether the increased metabolic rate, in the absence of the thyroid, is due to the same or

TABLE III. Increased Calorigenesis during Administration of a Pituitary Erythropoietic Fraction* in Rats Deprived of Functional Thyroid Tissue.

Group	Days after onset	No. of rats	Metabolic rate Cal/m²/hr	Metabolic rate % of normal	Red cell vol mg/100 g BW	Red cell vol % of normal	Terminal I^{131} uptake (5) thyroid region
Inj.	0	6	25.2 ± 1.3†	57	1.87 ± .05	81	—
	14	5	34.2 ± 1.8	78	2.18 ± .03	95	—
	21	5	38.2 ± 1.9	87	2.25 ± .03	98	.003
Uninj.	0	6	24.8 ± 1.3	56	1.84 ± .05	80	—
	14	6	26.0 ± .1	59	1.84 ± .05	80	—
	21	6	25.5 ± .5	58	1.80 ± .05	78	.003
Controls	21	15	44.0 ± 1.0	100	2.30 ± .05	100	7.5 ± .3

* 100 μg daily for 21 days of Preparation II-H-109-3.

† $SE = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

a different substance from that which stimulates erythropoiesis.

Clinical observations have supported the concept that metabolic rate does not depend entirely on the status of thyroid function. A high basal metabolic rate is usually observed in leukemic patients, whereas a normal I^{131} uptake by the thyroid of such patients has been noted(17). On the other hand, in the so-called hypo-metabolic state, where the basal metabolic rate is subnormal, there is no other evidence of decreased thyroid function (18).

Summary. 1. Erythropoietically active pituitary preparations increased the metabolic rate of hypophysectomized rats to normal levels with little or no stimulation of the thyroids. 2. A potent thyrotrophic preparation was less effective in increasing oxygen consumption than the erythropoietic factor. 3. The erythropoietic factor increased the caloric output to normal levels in rats in which all functional thyroid tissue had been destroyed.

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