It is concluded that the *in vitro* rumen fermentation technique can be used as a screening tool for detecting at least part of the metabolic inhibitor(s) in various fractions of orchardgrass.

Summary. Cellulose digestion determined by the *in vitro* rumen fermentation technique was used as a criterion to detect the presence of metabolic inhibitor(s) in various fractions of orchardgrass. The metabolic inhibitor is extractable with hot detergent, hot alkali-detergent, and azeotropic solution of methanol and acetone. The inhibitor appeared to be more concentrated in relatively low cellulose-digestibility orchardgrass than relatively high cellulose-digestibility orchardgrass.

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Effects of Erythropoietin on ^{14}C-Formate Uptake by Spleen and Bone Marrow Nucleic Acids of Erythrocyte-Transfused Mice.* (31918)

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It has been shown that erythropoiesis is almost completely suppressed in spleen of erythrocyte-transfused mice(1). ^{14}C-formate incorporation into the RNA and DNA of spleen of these animals is markedly depressed, while in marrow only a slight decrease is observed(2). A single injection of erythropoietin induces a wave of erythropoiesis that proceeds in an orderly fashion from proerythroblast to reticulocyte in the spleen of mice made polycythemic by transfusion(3). With such a system it is thus possible to study the incorporation of precursors into nucleic acids at different stages of erythropoiesis, at various times after erythropoietin injection.

It has been suggested that erythropoietin (EP) acts on the erythropoietic mechanism by stimulating an early synthesis of RNA (4,5). The experimental basis for this has

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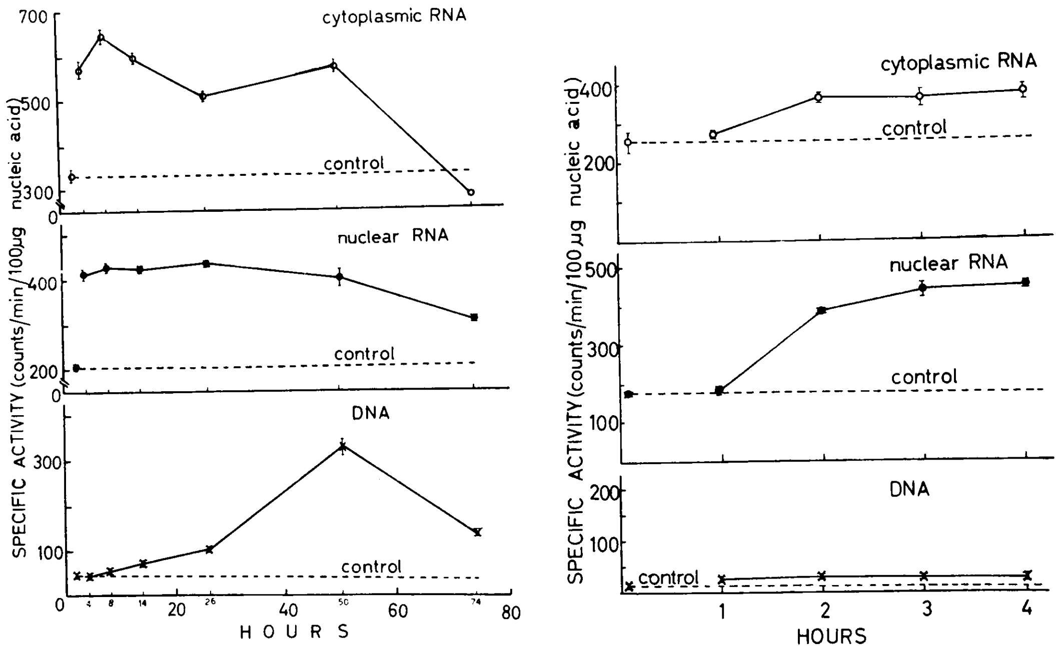

FIG. 1. Effect of a single dose of 5 u of erthropoietin on ^{14}C-formate incorporation into the cytoplasmic and nuclear RNA and DNA of spleen of erythrocyte-transfused mice. Figures give specific activity (mean ± S.E. of the mean). Base lines represent polycythemic controls. Specific activities of spleens of normal animals were 616 ± 70 (Cyt. RNA), 684 ± 95 (nRNA) and742 ± 44 (DNA). Haemoglobin concentrations were 13,8 - 15,6 (normal) and 21,3-23,3 9/100 ml (transfused). Formate was injected 2hr before sacrifice.

FIG. 2. Effect of a single dose of 5 u of erythropoietin on ^{14}C-formate uptake by the cytoplasmic and nuclear RNA and DNA of spleen of erythrocyte-transfused mice. Figures give specific activity (mean ± S.E. of mean). Baseline represent polycythemic controls. Specific activities of spleens of normal animals were 450 ± 59 (Cyt.RNA), 509 ± 89 (nRNA) and 265 ± 26 (DNA). Haemoglobin concentrations were 13,1 - 15,8 (normal) and 21 - 24,2 9/100 ml (transfused). Formate was injected 1hr before sacrifice.

been obtained however, in cultures of rat marrow(5) and in normal rats *in vivo*(4) under conditions where erythroid tissue was present. Since it is possible that EP as well causing differentiation of stem cells, may stimulate hemoglobin synthesis by recognizable erythroid precursors(6,7,8,9), it was considered important to evaluate the effect of erythropoietin on ^{14}C-formate, uptake in DNA and RNA, of marrow and spleen of erythrocyte transfused mice, in which erythroblasts are absent and one is presumably studying only the differentiation effect.

Material and methods. Male mice (C_{57} X C3 HF 1), weighing 25-30 g were obtained from the Department of Biology, Medical School, University of Chile. Polycythemia was produced by injecting intraperitoneally, on 2 successive days, .05 ml per gram body weight of isologous heparanized blood. Hemoglobin was determined as an index of the level of polycythemia(10) 5 days after the last blood transfusion. Then the animals were injected intravenously at time zero with 5 units of erythropoietin prepared from anaemic rabbit plasma by the method of Lowy and Borsook (11). The biological activity of the erythropoietin preparations was assayed in the fasted rat(12) against standard B from the National Institute for Medical Research, using a 4-point balanced parallel line design(13). 10 μc of ^{14}C-formate were injected intravenously at different intervals after EP injection and at least 3 mice were killed by a blow on the neck, either one or 2 hours after tracer injection, and the spleen femoral and bone marrow were removed for analytical treatment. Nuclear and cytoplasmic RNAs (nRNA and cRNA) were isolated and analyzed by the method of Smellie *et al*(14,15). The isolated RNAs and

TABLE I. ^{14}C-Formate Uptake by the Nucleic Acids of Bone Marrow of Erythrocyte Transfused Mice at Various Time Intervals Following a Single Injection of 5 Units Erythropoietin.

Group	Specific activity (counts/min/100 μg nucleic acid)*	
	Whole RNA	DNA
Normal mice	709 ± 64	1.152 ± 33
Control hypertransfusion polycythemic mice	416 ± 29	489 ± 58
Hypertransfusion polycythemic mice plus 5 units of erythorpoietin. Time of sacrifice after EP injection		
4	709 ± 20	569 ± 105
8	747 ± 79	643 ± 97
14	706 ± 6	670 ± 101
26	607 ± 60	855 ± 130
50	585 ± 39	824 ± 68
74	487 ± 67	461 ± 112

* Mean ± S.E. Marrow was obtained from the same animals used to measure spleen uptake (Fig. 1). Formate was injected 2 hr before sacrifice.

DNA were estimated directly in the solution after the digestion with 0.3 N KOH by the orcinol and diphenylamine techniques respectively(16). The ^{14}C-labelled nucleic acids were counted at infinite thinness in a gas-flow counter.

Results. Fig. 1 shows the effects of 5 units of EP on the specific activities of spleen nuclear and cytoplasmic RNA, and of DNA, at different time intervals after EP injection. ^{14}C-formate was injected 2 hours before killing the animals. Specific activities of nuclear and cytoplasmatic RNA are high after 4 hours. That of nuclear RNA remains so for 74 hours, while that of cRNA drops between 50 and 74 hours. Specific activity of DNA, is unchanged at 4 hours, begins to increase slowly after 8 hours and then more rapidly after 26 hours, to reach a maximum at 50 hours. Similar changes occur in marrow RNA and DNA (Table I). However as noted previously(2) depression of specific activities of marrow nucleic acids in control transfused mice is not as great as that observed in spleen.

To study the early effects of EP on ^{14}C-formate incorporation into RNA, experiments were carried out in which animals were killed at hourly intervals after EP injection. In these animals ^{14}C-formate was injected one hour before killing. Increase of specific activities of both nuclear and cytoplasmic RNA occurs between one and two hours after EP injection and specific activities reach plateau levels by 3 hours (Fig. 2). No significant changes occur in DNA specific activities during this time. Similar changes occur in bone marrow RNA (Table II).

Table III gives the results of control experiments in which the effects of EP inactivated by mild acid hydrolysis(17) were compared to those of the active EP preparation. Inactive EP does not produce an effect on the specific activity of spleen RNA.

Discussion. Injection of EP produces an increase in the specific activities of nuclear and cytoplasmatic RNA of spleen and marrow within 2 hours after injection in polycythemic

TABLE II. Effect of a Single Dose of 5 Units of Erythropoietin on ^{14}C-Formate Uptake by the Bone Marrow Nucleic Acids of Erythrocyte-Transfused Mice During the First Hours of EP Action.

Group	Specific activity (counts/min/100 μg nucleic acid)*	
	Whole RNA	DNA
Normal mice	634 ± 46	1.022 ±119
Control hypertransfusion polycythemic mice	328 ± 41	289 ± 55
Hypertransfusion polycythemic mice plus 5 units of erythropoietin. Time of sacrifice after EP injection		
1	352 ± 34	418 ± 81
2	422 ± 35	407 ± 36
3	516 ± 66	424 ± 86
4	508 ± 39	378 ± 3

* Mean ± S.E. Marrow was obtained from the same animals used to measure spleen uptake (Fig. 2). Formate was injected 1 hr before sacrifice.

TABLE III. Effect of Erythropoietin and Inactivated Erythropoietin Containing (IE) Extracts on Uptake of ^{14}C-Formate into the Nucleic Acid of Spleen of Erythrocyte-Transfused Mice.

	Specific activity (counts/min/100 μg nucleic acid)		
	Control	Inactivated erythropoietin	Erythropoietin
Nuclear RNA	182 ± 14	233 ± 24	361 ± 49
Cytoplasm. RNA	240 ± 39	261 ± 39	418 ± 20
DNA	35 ± 8	25 ± 2	33 ± 7

Mice killed 4 hr after erythropoietin injection, ^{14}C-formate injected 2 hr before sacrifice. The other conditions were similar to those described in text and Table I.

mice. This increase probably reflects increased incorporation of formate into RNA, since the total amount of RNA is not changed at this time. The fact that the specific activity of DNA measured simultaneously does not change, suggests that the increased incorporation is not due merely to a change in the specific activities of the purine bases, but reflects an increased synthesis of RNA. The specific activity of DNA of spleen marrow remains unchanged for 8 hours after EP injection, then rises slowly at first, and more rapidly after 26 hours, with a clear maximum at 50 hours in spleen. Kurtides *at al*(18) have shown that increased uptake of H^3 Thymidine in spleen of normal rats injected with EP, begins after 16 hours. The increase in labelled precursor uptake in DNA presumably reflects the increase of the number of erythroid precursors in S phase that occurs during the erythropoietic wave induced by EP(3,19).

The finding that the maximum formate uptake in DNA of erythropoietic tissue of transfused mice injected with 5 u EP is below that of normal mice, is in agreement with the results of our measurement of erythropoietic function with ^{59}Fe. While normal mice have a 24-hour erythrocyte ^{59}Fe uptake of 31.3 ± 0.9 hypertransfused mice injected with radio iron, 48 hours after 5 u EP, have a 24-hour erythrocyte ^{59}Fe uptake of 19.5 ± 1.4. Hypertransfused non EP injected mice have an uptake of 0.12 ± 0.03.

These studies carried out in transfused mice, where the differentiation effect of EP is being studied, indicate that in this system also, increase of nuclear RNA synthesis is the earliest observable effect of EP injection. That the effect is specific to EP and not due to contaminants in the extract, is suggested by the finding that the mild acid treatment, which inactivates EP(17) also makes it lose its effect on RNA synthesis.

Further studies characterizing the RNA that is produced under the influence of EP, will be necessary to find out what type of RNA is being produced, whether it be ribosomal precursor, messenger or transfer type, and whether the type of RNA produced changes with time after EP injection. The spleen of transfused mice seems well suited for these studies.

Summary. The effect of erythropoietin on the incorporation *in vivo* of ^{14}C-formate into the nucleic acid of spleen and bone marrow of erythrocyte-transfused mice has been investigated. Erythropoietin increases the specific activity of nuclear and cytoplasmic RNA between one and two hours after injection of the hormone. Specific activity of nuclear RNA remains high for 74 hours while that of cytoplasmic RNA drops between 50 and 74 hours after EP administration. Specific activity of DNA begins to increase slowly at 8 hours and then more rapidly at 26 hours after EP to reach a maximum at 50 hours. Experiments with inactive erythropoietin suggest that the effect on nucleic acid metabolism is due to the hormone and not to other substances contained in the erythropoietic preparations. In a condition such as that of erythroid tissue of the transfused mouse, where only the differentiating effect of EP is being studied, the earliest effect observed is an increase of the uptake of ^{14}C-formate into nRNA, followed later by increased uptake into DNA, which presumably reflects the increase of erythroid cells in S phase during the erythropoietic wave that follows EP injection. Further studies are required to determine the nature and significance of the RNA synthesized, soon after EP injection.

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Synthesis of RNA and DNA at Various Intervals After Erythropoietin Injection in Transfused Mice. (31919)

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Erythropoietin injection in hypertransfused mice induces a wave of erythropoiesis in the spleen(1). ^{59}Fe incorporation into Hem does not occur until 24 hours after erythropoietin (EP) injection in this assay system(2), while immediate increase of ^{59}Fe uptake is observed in marrow cultures on addition of EP(3). Increase of ^{14}C-uridine incorporation into cultured marrow RNA is seen within 20 minutes of EP addition(4) and of ^{14}C formate in rat marrow RNA *in vivo,* within 4 hours of EP injection(5). Since there is marked difference in time of response between the transfused mouse spleen and rat marrow "in culture" and *in situ* in relation to hemoglobin synthesis, it was considered of interest to establish the time course of the RNA, DNA synthesis in the spleen after EP injection in hypertransfused mice.

Methods. Three-months-old 20 g female CBA mice were used. Transfusion erythrocytosis, with Hb concentrations greater than 18 g/100 ml, was produced by intraperitoneal injection of 1 ml of whole CBA blood, per 20 g on days zero and one. Erythropoietin 1 mg of a 60-80% ethanol fraction of anemic rabbit plasma(6) equivalent to 5 U St.B, was injected on day 6 and the following tracers obtained from the Radiochemical Center, Amersham, were injected at times indicated in *Results:* uridine 5-^{3}H 8 μc/mouse, iv., thymidine -^{3}H (methyl) 8 μc/mouse, i.v.

Groups of at least 5 mice were killed 5 minutes after ^{3}H uridine and 60 minutes after ^{3}H thymidine injection. Spleens were removed and immediately frozen in dry ice-ethanol. The nucleic acid fraction was prepared from a water homogenate by the method described in(7). The dry powder obtained was dissolved in 1 ml hyamine and counted in POP, POPOP toluene phosphor in a TriCarb liquid scintillation spectrometer. An internal ^{3}H toluene standard was used for quench corrections. Samples were kept in the dark and cold for 48 hours before counting (8).

Results. Uridine uptake in rapidly labelled RNA increases 2 hours after EP injection and remains high for 60 hours (Fig. 1). Thymidine uptake does not rise until after 12 hours and shows 2 maxima at 24 and 48 hours separated by a dip at 36 hours. By 60 hours it is back to control levels. Control experiments with EP inactivated by mild acid treatment(9) showed no increase of thymidine uptake at 24 hours and of uridine at 6 hours after EP (Table I).

Discussion. It is known that 5-^{3}H-uridine

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