Table I. Distribution of radioactivity 2 h after i.v. injection of sodium acetate-^{14}C with varying amounts of EDTA

Na_2Ca-EDTA (μmol)	Percentage of injected ^{14}C-dose [a] Plasma (1 ml)	Liver	Kidneys	Muscles [b]	Skeleton [c]	Labile skeletal fraction [d]
0	0.22 ± 0.01	3.63 ± 0.97	0.70 ± 0.07	9.65 ± 1.96	3.14 ± 0.32	1.12 ± 0.10
1	0.21 ± 0.03	4.57 ± 2.17	0.82 ± 0.06	9.87 ± 0.59	2.99 ± 0.18	1.02 ± 0.07
100	0.22 ± 0.02	3.09 ± 1.09	0.71 ± 0.11	8.66 ± 1.99	3.19 ± 0.12	1.02 ± 0.11
400	0.19 ± 0.02	2.28 ± 0.59	0.69 ± 0.15	7.62 ± 1.02	2.60 ± 0.37	0.91 ± 0.19

[a] Arithmetic means and 95%-fiducial limits. 5 rats per group. [b] Total muscle mass was assumed to equal 40% of body wt. [c] ^{14}C in one femur times 20. [d] Radioactivity released as $^{14}CO_2$ after treatment of femur with hydrochloric acid[2].

exception of the kidneys, there is no statistically significant influence of the lowest dose of EDTA on the accumulation of ^{14}C. With higher doses of EDTA, however, the retention of ^{14}C in the liver, kidneys and muscles decreases and regression lines may be calculated when plotting the experimental values against log EDTA dose. The estimated slopes of regression lines (Table II) in liver and muscles are virtually identical, while the ^{14}C content of the kidneys decreases more slowly. The linear terms of the regressions are significant. However, this correlation does not exist for the skeletal retention of ^{14}C, although this is significantly lower when 400 μmol instead of 100 μmol EDTA are added. Approximately one-third of the skeletal ^{14}C can be released by treatment with hydrochloric acid (Table I), irrespective of the amount of EDTA injected.

Our data are in favour of the above-mentioned hypothesis, i.e. they demonstrate a definite influence of EDTA on the distribution pattern of radioactivity after the injection of ^{14}C-labelled acetate. This might suggest the effect of EDTA on the metabolic turnover of acetate in soft tissues rather than the dose-dependent deposition of EDTA itself. The accumulation of ^{14}C in the skeleton is much less affected, and the loss of radioactivity in the bone after acid treatment indicating incorporation of ^{14}C into the labile carbonate pool in bone[5] is independent of EDTA. Since our knowledge of other than morphological effects of the chelating agents is extremely poor, further studies are required to show whether our observation on the effect of EDTA might be useful in evaluating the physiological and/or toxic action of the chelates[6].

Table II. Accumulation of ^{14}C in relation to log dose of stable EDTA

	Regression coefficient ± S.E.	Significance (P)
Liver	−0.844 ± 0.262	0.01
Kidneys	−0.053 ± 0.006	0.025
Muscles	−0.795 ± 0.247	0.01
Skeleton	−0.088 ± 0.071	0.30

Zusammenfassung. Die ^{14}C-Ablagerung nach i.v. Injektion von Ratten mit ^{14}C-markiertem Natriumacetat nimmt in Leber, Nieren und Muskeln mit steigender Zugabe (1–400 μmol) von gleichzeitig injiziertem Na_2[Ca-ÄDTA] ab. Hiermit wird ein bisher noch nicht beschriebener Effekt vom Chelatbildner nachgewiesen.

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Effect of Erythropoietin on the Activity of RNA Polymerase

The first molecular action of erythropoietin known until today is the early stimulation of the metabolic activity of a nuclear RNA of different characteristics[1,2]. These findings have suggested the idea that the primary site of action of the hormone may be at some level in the transcription stage[3]. To explore this possibility it seemed reasonable to study the activity of RNA polymerase under the influence of the hormone. Under these conditions the stimulation of the enzyme could be interpreted as the result of a direct control of the hormone on the RNA metabolism. This paper describes experiments that show the modifications produced in the activity of RNA polymerase in isolated nuclei from rat bone-marrow treated with erythropoietin.

Material and methods. Male rats of the strain A × C, weighing 150–170 g, were used. The cell nuclei were obtained from bone-marrow cells of normal and erythropoietin treated rats. The latter were collected from rats that received an i.v. injection of 5 units of erythropoietin. Bone-marrow cells were extracted from the femur and tibia and then homogenized in 10 vol. of cold 0.25M sucrose that contained $10^{-3}M$ $MgCl_2$. The homogenate

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was sedimented at 750 *g* for 10 min at 0°. The pellet was resuspended in 2.2 *M* sucrose with 10^{-3} *M* $MgCl_2$ and then centrifuged at 43,500 *g* for 90 min at 0°. The cell nuclei pellet was purified according to the method described by CHAUVEAU et al.[4]. The nuclei preparations were checked under a phase microscopy and they were found free of cytoplasmic contaminants.

The incorporation of (8-^{14}C)-ATP into the RNA was taken as a measurement of polymerase activity. Unless indicated otherwise each test-tube contained 0.4 μmoles each of ATP, GTP, CTP and UTP; 0.1 μC of (8-^{14}C)-ATP; 0.2 ml of the nuclei suspension in a final volume of 0.4 ml at pH 6.5. The test-tubes were maintained at 37 °C for variable periods of time. The reaction was stopped by the addition of saturated sodium pyrophosphate, 0.5 ml of serum albumin and 1ml of 2.1 *N* perchloric acid. The precipitate was separated by centrifugation and then the RNA was isolated by a method already described[5]. The preparation of a soluble form of the DNA-dependent RNA polymerase from rat bone-marrow nuclei was done by the method described by CUNNINGHAM and STEINER[6].

The amount of RNA isolated in the form of nucleotides was estimated by the orcinol reaction or by measuring the UV-absorption with a 450 Perkin-Elmer spectrophotometer. The radioactivity was counted in a Nuclear Chicago gas-flow counter. DNA was measured by the reaction of diphenylamine[7] and protein according to the method of LOWRY[8]. Results are expressed as specific activity in count/min/mg of DNA or of proteins.

Results. The Figure shows the in vitro activity of the RNA polymerase of isolated bone-marrow cell nuclei of control and erythropoietin treated rats. The incubation time course of the nuclear preparations shows that the activity of the RNA polymerase obtained from the rats treated with the hormone is nearly 3 times higher than from the control. After 20 min of incubation, the activity of both nuclear fractions decreases and this is probably due to the poor conditions of the incubation medium.

Some of the characteristics of the RNA polymerase activity are illustrated in the Table. Notice that the enzyme activity is enhanced by the presence of Mn^2 ion, mercaptoethanol and ammonium sulphate while the incorporation of the labelled ATP is diminished when actinomycin D or DNase are added to the incubation mixtures. The requirement for the 4 ribonucleoside triphosphate seems not to be essential for this kind of nuclear preparations. The activity of the enzyme with the addition of ATP only is probably due to the existence of a large RNA precursor pool in the marrow cell nuclei. The properties described for the bone-marrow RNA polymerase are in general in agreement with those reported to other RNA polymerase preparations obtained from several mammalian tissues[6,9,10].

To test whether the action of the erythropoietin on RNA polymerase activity is a direct effect, a preparation of the enzyme in a soluble form was carried out[6]. Experiments have demonstrated that the activity of this RNA polymerase is slightly increased by the hormone in vivo, while it has not been possible to detect any effect of the erythropoietin on the enzyme activity in vitro. In other words, the hormone does not produce a direct augmentation of the enzyme activity.

The presence in the incubation media of all of the 4 ribonucleosides triphosphate is required for the soluble preparation. Thus, the omission of 3 of them produces threefold decrease in the specific activity of the enzyme. However, no decrease in the specific activity of the enzyme is observed when the same experiments are repeated with the nuclear fraction containing the enzyme as it is shown in the Table.

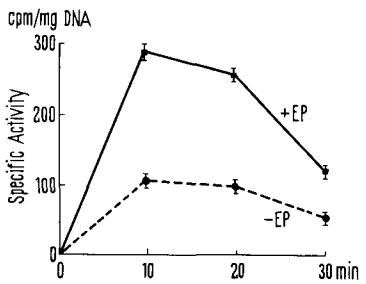
Discussion. The properties of the RNA polymerase preparations are in agreement with the current thought that defines the conditions for RNA polymerase activity. However, to analyze more critically the properties of the bone-marrow RNA polymerase, it would be necessary to isolate the enzyme in a more purified form. This is beyond the intention of this communication.

The different response of the enzyme in in vivo and in vitro conditions under the influence of the hormone seems to indicate that the erythropoietin does not appear directly to enhance the enzyme activity suggesting the idea that the hormone might act in a molecular reaction that heads the action of RNA polymerase. It is possible

Some properties of the RNA polymerase action on isolated bone-marrow nuclei

Experimental conditions	Specific activity (counts/min/mg DNA)
Complete system (CS)	430 ± 22
CS minus GTP, CTP, UTP	366 ± 13
CS plus 0.6 m*M* $MnCl_2$	560 ± 70
CS plus 5 m*M* mercaptoethanol	585 ± 10
CS plus 0.05 *M* ammonium sulphate	520 ± 35
CS plus 0.05 *M* ammonium sulphate and 5 m*M* mercaptoethanol	778 ± 88
CS plus 100 μg of actinomycin D[a]	206 ± 10
CS plus 100 μg of DNase[a]	199 ± 14

[a] Preincubated for 30 min. The experimental conditions are indicated in the text. The results are of a typical experiment from a series of 3. The Figures give specific activity (mean of 3 samples ± S.E.).



The effect of erythropoietin on the RNA polymerase activity. The nuclear fractions were obtained from the bone marrow of normal rats (broken line) and from normal rats i.v. injected with 5 U of erythropoietin (EP) for 4 h (solid line). Points represent average values from 8 determinations. Bars indicate the standard error of the mean value. The experimental conditions are indicated in the text.

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that erythropoietin binds to the chromatin, probably by a protein-protein bound and then renders a portion of the DNA available as a template for the synthesis of RNA. In vivo stimulation of the RNA polymerase may also be explained in this manner. PINTO[11] described the formation of a complex between erythropoietin and DNA from tissues like bone-marrow, liver, spleen and thymus. The binding occurs in a particular region of the DNA and probably involves a potein-protein interaction in which the hormone could play the role of an allosteric ligand in respect to the other protein.

In a recent paper GROSS and GOLDWASSER[1] have reported that shortly after erythropoietin is made available to marrow cells in vitro, a very large RNA (150 S) is synthesized. This RNA cannot be detected in control samples without hormone. The 150 S RNA has a short half-life of approximately 6 min. All the data presented suggest the following sequential molecular events induced by the erythropoietin.

The first molecular action of the hormone would be related to a specific binding to a protein receptor at the chromatin level. As a result of this action, a specific region of the DNA is susceptible to be used as a template. The second molecular event is related to the action of the RNA polymerase and the synthesis of a 150 S RNA. The following step may be related with the degradation process of the 150 S RNA and the preparation of the biochemical units for the protein synthesis.

The sequential nuclear reactions discussed above are consistent with the idea that the information for the synthesis of haemoglobin must be prepared during the early stages of the erythropoietic process[12,13]. The evidence presented firmly supported the hypothesis that the erythropoietin acts at molecular level in the transcription stage. Further studies of this idea are in progress and will be reported later on[14].

Resumen. La eritropoyetina estimula in vivo la actividad de la RNA polimerasa de médula ósea de rata. In vitro, la hormona no parece aumentar la actividad de la enzima. Las características de la acción enzimática demuestran que la actividad medida corresponde a la de la RNA polimerasa descrita en otros tejidos. Se sugiere que el sitio primario de acción de la hormona está en la etapa de transcripción y se discuten los eventos moleculares que produce la eritropoyetina a este nivel.

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Role of Phosphate and Acid Phosphatase During Germination of *Pinus pinea* Seed[1]

A striking feature of seed germination can be considered to be the mobilization of storage energetic material accumulated in reserve tissues, and the following translocation and utilization of the same for the development of embryo axis. Phosphate plays an extremely important role in a variety of reactions in germinating seed, where it appears primarily in a linked organic form and very little seems to be present as free inorganic orthophosphate[2], so that it represents a limiting factor in many reactions.

Correlated with this fact, the increase in activity of typical hydrolitic enzymes as phosphatases appears particularly significant and the change in free and total phosphate level observes in the same period. Researches on this biological mechanism have been made extensively during the life cycle of algae[3], fungi[4] and in germinating seed of some angiosperms[5,6]. Recently an extensive plan of research on the metabolism of Gymnosperms led us to investigate the alternance of metabolic pathways during seed germination of different species of conifers[7–10] but our data are lacking in the role of phosphate in this systematic group.

In this paper we have followed the time sequences of the changes in free and total phosphate compared with the levels of acid phosphatase in seedling and in the endosperm, root and shoot of germinating seed of *Pinus pinea*.

Experimental. Seeds of *Pinus pinea* were cultivated on moistened sand at 20 °C for a 9 h photoperiod. Preparation of extracts and determination of acid phosphatase, free and total phosphate and protein content was performed as previously indicated[6].

Results and discussion. Results are reported in Figures 1 (a, b, c, d) and 2, where the values of acid phosphatase, free and total phosphate are reported during the first 20 days of germination in different parts of germinating seed. In general total phosphate remains constant in the experi-

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