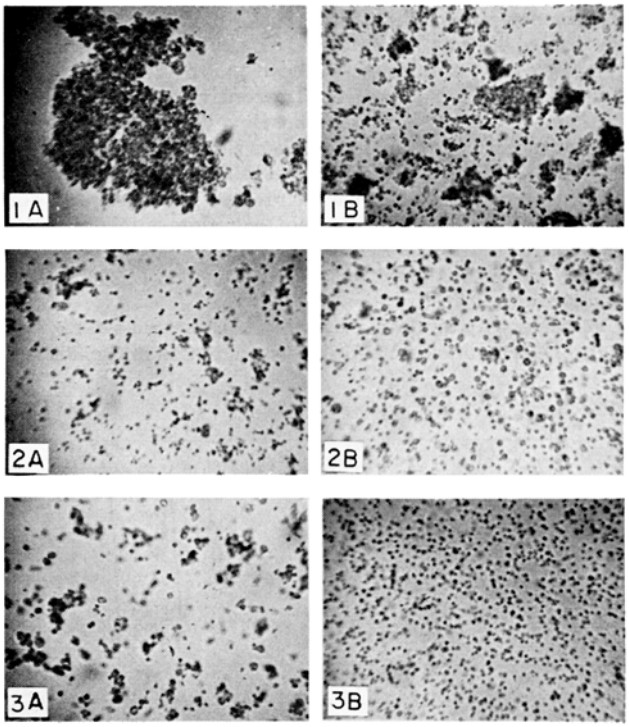
Agglutination of prednisolone treated cells by wheat germ agglutinin

Agglutinin concentration (ml/ml cell suspension)	Agglutination reaction			
	Henle		HeLa S3	
	Prednisolone	Control	Prednisolone	Control
0.100	++++	+++	++++	+++
0.075	+++	+++	+++	++
0.050	+++	+++	+++	++
0.025	++	++	++	+
0.010	++	++	+	0
0.005	+	0	+	0
0.000	0	0	0	0



Effects of neuraminidase on the agglutination of HeLa S3 cells by wheat germ lipase. Photographs 1A, 2A and 3A cells grown in the presence of prednisolone; 1B, 2B and 3B cells grown without prednisolone; 1, wheat germ agglutination (0.025 ml/10^6 cell suspension), 2 same as 1 but pretreated with 5 units of neuraminidase, 3 control suspension.

suspended in saline to a density of 1×10^6 cells/ml. Wheat germ lipase purchased from Sigma Chemical Company was treated according to the procedure described by BURGER and GOLDBERG[4]. The homogenized enzyme preparation was heated at 63°C for 15 min and centrifuged. The clear supernate was used as the source of agglutinin. No further purification of the preparation was attempted. The agglutination assay was performed according to the procedure of AUB, TIESLAU and LANKESTER[3] and scored as described in their paper.

Results of one such experiment are shown in the Table. They indicate that pretreatment with prednisolone resulted in a qualitative and quantitative change in the agglutination pattern. In both cell lines agglutination in steroid treated cells was considerably stronger, moreover it was detectable at higher dilutions of the wheat germ agglutinin. In order to check the effect of neuraminidase on cell agglutination, the cells were treated before addition of agglutinin for 1 h at 37°C with purified neuraminidase from *Clostridium perfringens* (5 units/10^6 cells). As shown in the Figure the agglutination of both steroid-treated and control cells was abolished by the treatment with neuraminidase.

At the present time it is too early to speculate upon the significance of these results. As suggested by KRAEMER[8] the increased sialic acid content reflects an increase in the surface area of the cells which is also one of the results of steroid treatment. On the other hand, prednisolone could modify the structure of the cells in such a way as to expose more sialic acid groups to the action of agglutinin[9].

Zusammenfassung. In menschlichen Zellkulturen mit Zugabe von Prednisolon wird die Agglutinierbarkeit der Zellen durch den Einfluss der Weizenkeimlipase gesteigert. Die Vorbehandlung mit Sialidase (*N*-Acetylneuraminidase) verhindert die Agglutination, was auf eine Hormonwirkung an der Zellmembran hinweist.

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Effect of Erythropoietin on Red Cell Differentiation Binding of Erythropoietin to DNA

It is now well established that erythropoietin is involved in red cell differentiation[1]. Although its mechanism of action is not fully understood, the outstanding work of KRANTZ et al.[2] suggests that its mode of action can be explained through the genetic activation hypothesis postulated by MONOD and JACOB[3]. Such a hypothesis implies, (1) identical structure of the DNA in all the cells from different tissues within an organism, (2) permanent repression of a given region of the DNA through the different stages of the development of such organism, and (3) removal of the repressor in a particular cell by the differentiation factor or corepressor while the same region in the rest of the cells remains unaffected. In the case of

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erythropoietin, its mechanism of action as a corepressor is mainly based on 2 facts, (1) stimulation of the synthesis of an apparent m-RNA, and (2) blocking of erythropoietin stimulation by the administration of actinomycin[2]. The purpose of this paper is to present evidence that erythropoietin can bind to the DNA from certain tissues and that this is possibly related to its mechanism of action.

Methods. Sheep erythropoietin purified by the procedure of GOLDWASSER et al.[4] having a specific activity of 22 cobalt units/mg of protein was assayed by the rate of incorporation of Fe^{59} into the haem molecule when bone marrow cells were grown in tissue culture by a similar procedure described by KRANTZ et al.[5]. Mixtures, sterilized by filtration through 0.45 μ pore filter, contained, in a final volume of 2.5 ml; 5 μC of Fe^{59}, 2.25 ml of NCTC-calf serum (50:50), 250 units of penicillin, 15×10^6 bone marrow nucleated cells from 200–250 g Sprague-Dawley rats and sample. Cultures were incubated in 30 ml polystyrene flasks in a National water jacketed incubator at 37°C under an atmosphere of 5% CO_2-air. At different periods of time, usually 40 h, incubations were stopped by centrifugation at 4,000 rpm for 15 min in a portable centrifuge. The precipitates obtained were each washed twice with 2 ml of 0.9% NaCl and the resulting suspensions centrifuged as mentioned. Porphyrins were extracted from the precipitated cells by shaking them overnight at 4°C with 5% (v/v) HCl-acetone. The debris was removed from the extracts by centrifugation in a SM24 Sorvall head in a RC-2B Sorvall centrifuge set to 4°C and 15,000 rpm for 1 h. Most of the Fe^{59} (98–99%) was contained in the haem which was identified by paper chromatography[6] followed by autoradiography. The radioactivity contained in the Fe^{59} haem was measured by placing aliquots of the above mentioned extracts in aluminum planchets in a 151 A Nuclear Chicago end window counter. Porphyrins in the extracts were determined by measuring the absorption at 400 nm using equine haematin III as standard[7]. DNA was extracted from the brain, bone marrow, kidney, liver, spleen and thymus of Sprague-Dawley rats (200–250 g body weight) by the method of KIRBY[8]. The fibrous precipitate containing DNA and obtained by this procedure was dissolved in the minimum volume of 40 m*M* *Tris*-Cl (pH 7.4) containing 10 m*M* $CaCl_2$. To this mixture, 1 mg of trypsin, (purified according to NORTHROP et al.[9]) was added and the whole incubated at room temperature for 6 h. Following incubation the DNA was again extracted by the method of KIRBY. Samples of 0.2 ml of the DNA (0.2 mg) obtained from the different organs, together with 5 cobalt units of erythropoietin, DNA alone or erythropoietin alone were applied on the top of 4.6 ml linear sucrose density gradients of densities ranging from 1.01 at the top to 1.10 at the bottom. The tubes containing the gradients and samples were placed in a S.W. 39 head and centrifuged in a Spinco L-4 ultracentrifuge set at 38,000 rpm and 4°C for 10 h. After centrifugation the bottoms of the tubes were pierced, 30 fractions were collected and the 260 nm absorption in each fraction determined. After dialysis against water each fraction was assayed for erythropoietin activity. Protein was determined by the procedure of LOWRY et al.[10] and amino acids with ninhydrin[11]. All the operations were performed at 4°C unless otherwise indicated.

Results. All the data presented are mean of 2 experiments. Erythropoietin activity was detected in the 260 nm

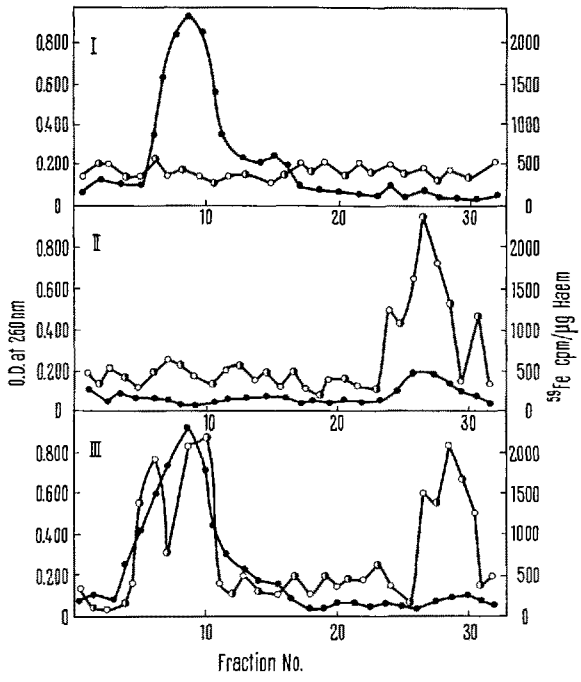

Fig. 1. Binding or erythropoietin to DNA from bone marrow. Sucrose density gradient centrifugations containing bone marrow DNA (I) erythropoietin (II) and bone marrow DNA plus erythropoietin (III) were performed as described in 'Methods'. Hormone activity assays before (o) and after being treated with DNAase (◑) were determined as described in the text. The same phenomenon was observed with DNAs from liver, spleen and thymus. DNA (●) was measured by the 260 nm absorption.

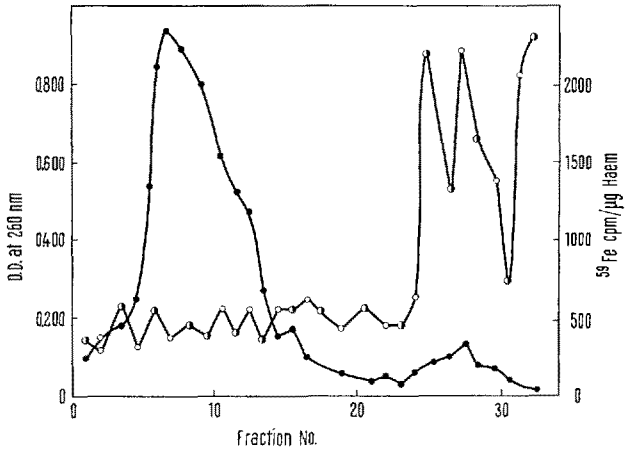

Fig. 2. Lack of binding of erythropoietin to DNA from brain. Experimental procedure as described in Figure 1.

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absorbing peak of the DNA from bone marrow, liver, spleen and thymus (Figure 1). However, no activity was found in this region when the DNA used came from brain and kidney (Figure 2). An additional peak of erythropoietin activity was found in the region corresponding to free erythropoietin (Figure 1). The presence of DNA in the 260 nm absorbing peak was shown by the disappearance of the absorption after the treatment of each fraction with 0.02 mg of bovine pancrease DNAase (purified by the procedure of KUNITZ[12]) for 16 h and 4°C followed by thorough dialysis against water. No effect of the DNAase and DNA peak on the erythropoietin activity was found (Table I). Erythropoietin activity from apparently bound and free peaks disappeared after treatment of each fraction with 2 μg of trypsin in 40 m*M* *Tris*-Cl (pH 7.4) and 10 m*M* $CaCl_2$ for 2 h at room temperature. The enzyme reaction was terminated by chilling the corresponding fraction in crushed ice followed by thorough dialysis against water. No detectable protein was found in any of the DNA samples. Similarly, following trypsin digestion no amino acids could be detected in the dialyzate. Experiments where denatured DNA was used for the exploration of the binding properties of erythropoietin to DNA were unsuccessful.

Table I. Effect of brain and kidney DNAs on erythropoietin activity

	Fe^{59} cpm/μg Haem
Control	8,600
Control after DNAase digestion	9,750
Brain DNA pool	
0.1 ml	9,150
0.2 ml	8,750
Brain DNA pool after DNAase digestion	
0.2 ml	10,200
Kidney DNA pool	
0.1 ml	8,300
0.2 ml	8,850
Kidney DNA pool after DNAase digestion	7,500

Main fractions corresponding to pools from brain and kidney 260 nm absorbing peaks from a similar experiment to Figure 2 were tested for erythropoietin activity as described in 'Methods' after the addition of 0.2 cobalt units of erythropoietin. DNAase digestion was performed as described in the text.

Table II. Effect of trypsin digestion on the erythropoietin activity

	Fe^{59} cpm/μg Haem
Control without erythropoietin	850
Control after trypsin digestion	930
Bound erythropoietin	2,620
Free erythropoietin	4,600
Bound erythropoietin after trypsin digestion	760
Free erythropoietin after trypsin digestion	1,120

Pooled fractions containing free and bound erythropoietin-bone marrow DNA from a similar experiment to that shown in Figure 1 were incubated with trypsin as described in the text and then erythropoietin activity determined according to 'Methods'. DNAs from bone marrow, and spleen were tested.

Discussion. The results described in this paper show that the ability of erythropoietin to bind to DNA depends to a large extent upon the tissue source of DNA. While erythropoietin apparently binds to the DNA extracted from bone marrow, liver, spleen and thymus, a similar behaviour was not shown by the DNA of brain and kidney. It is attractive to suggest that small chemical differences exist in the DNA extracted from different tissues and which were responsible for the binding or erythropoietin to certain of the DNAs. However, it is also possible, that the preparations of DNA which bound erythropoietin were impure and that it was the impurity which was responsible for the binding. From the extraction procedure it would seem that the most likely contaminant would be protein, however, no protein was detected in any of the DNA preparations and no amino acids were found in the dialyzate following dialysis of the trypsin digestions. It would seem likely, then, that differences do exist in the DNA extracted from different tissues and that these differences are responsilbe for the distinct behaviour of some DNA preparations in binding erythropoietin. These differences must be very small since the neutralization binding of DNA from one mammalian source to that of another is always the same[13]. Since all DNA within an organism initially comes from the same origin these differences must have originated during the development of the organism. It is suggestive therefore, that erythropoietin and similar factors could be involved in this process through the binding to a particular region of the DNA. Such a binding would imply that erythropoietin is capable of stimulating cell division, DNA synthesis and also able to modify the message contained in the DNA at the time it is transferred to the daughter cells. Verification of this hypothesis would require a study of the physico chemical properties of the different fractions of the DNA isolated by conventional methods. The binding properties which erythropoietin possesses towards DNA extracted from certain tissues could then be used as a marker to identify these DNA portions with this characteristic[14].

Zusammenfassung. Die Fähigkeit der DNS verschiedener Rattengewebe, Erythropoietin zu binden, wurde durch Differentialzentrifugation im Sucrosegradient untersucht. Es ergab sich, dass DNS von Knochenmark, Leber, Milz und Thymus Erythropoietin binden, DNS von Hirn und Nieren Erythropoitein hingegen nicht zu binden vermögen.

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