EXHIBIT B

Lodish Decl. in Support of Opposition to Roche's Motion for Summary Judgment of Invalidity for Double Patenting Over Claim 10 of the '016 Patent

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On the Mechanism of Erythropoietin-induced Differentiation

XIII. THE ROLE OF SIALIC ACID IN ERYTHROPOIETIN ACTION*

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SUMMARY

Erythropoietin, a glycoprotein that induces normal erythrocyte development, has 16 to 18 sialic acid residues per mole. Desialation results in complete loss of biological activity when it is assayed *in vivo*. When the assay is done *in vitro* asialoerythropoietin has full activity, or when assayed at low levels of hormones is about three times more active than the native hormone. The loss of activity can be explained by the hepatic removal of asialoglycoproteins from the circulation (Morell, A. G., Irvine, R. A., Sternlieb, I., Scheinberg, I. H., and Ashwell, G. (1968) *J. Biol. Chem.* 243, 155). Asialoorosomucoid or stachyose injected into assay animals act as competitors and permit 25 to 35% of the original activity of erythropoietin to be seen *in vivo* after desialation. Treatment of asialoerythropoietin with galactose oxidase results in recovery of 45% of the original activity, confirming an earlier finding (Lukowsky, W. A., and Painter, R. H. (1972) *Can. J. Biochem.* 50, 909). Asialoerythropoietin is more sensitive to heat denaturation and trypsin action than the native hormone. Our data indicate that terminal sialic acid is required for chemical and biological stability but not for action on target cells of the bone marrow. The asialohormone has enhanced activity towards the target cells.

It has been known for some time that the role of sialic acid in the biological function of erythropoietin is paradoxical. Erythropoietin activity, when measured *in vivo*, is completely dependent on the presence of sialic acid (2), but when the assay is done *in vitro*, the asialohormone has full activity (3). Resolution of this apparent contradiction was facilitated by the findings of Morell *et al.* (4) who showed that, in general, asialoglycoproteins disappear from the circulation of animals at a much greater rate than do the corresponding sialic acid-containing proteins. This increased rate of loss is due to hepatic cell recognition of newly terminal galactose residues and subsequent binding of the asialoglycoprotein (5). The observations of Morell *et al.* suggested that asialoerythropoietin might behave *in vivo* in a similar fashion and hence would have little opportunity of productive interaction with target cells of the hemopoietic system. *In vitro*, however, where there are no hepatic cells, asialoerythropoietin could affect the target cells.

If the findings with other glycoproteins can be generalized to include erythropoietin, it would be expected that modification or removal of the putative galactose residues, made terminal by desialation, would result in restoration of activity *in vivo*. It would also be expected that pretreatment of assay animals with competitive compounds having terminal galactose residues would permit asialoerythropoietin to exert an effect *in vivo*. Lukowsky and Painter (6) have shown that oxidation of partially desialated erythropoietin resulted in restoration of some *in vivo* activity. In the present paper we confirm that finding with the use of fully desialated erythropoietin. We also demonstrate that the use of competitors results in partial restoration of *in vivo* activity. In addition, we have found that removal of sialic acid from erythropoietin causes diminished stability of the hormone and an increase in its activity measured *in vitro*.

MATERIALS AND METHODS

Bioassay—The fasted rat method of bioassay (7, 8) was used throughout this study. Some modifications of this method, made since it was originally published, are as follows. Male Sprague-Dawley rats, 3 months old, were stored for 1 week before use. Only rats weighing between 215 and 245 g after the week of storage were used. Samples were injected by tail vein and radioiron by the intraperitoneal route.

Each assay consisted of four groups of rats receiving standard doses of erythropoietin (1.0, 1.5, 2.0, and 3.0 units per rat), one control group which received 0.1% bovine serum albumin in 0.15 M NaCl, and the test groups. There were five rats in each group. The response measured was the percentage of incorporation of ^{59}Fe into the total red cell mass with the control value subtracted. The standards were fit to a ln units *versus* ln response curve by regression analysis, and the test group unitage values were determined from the slope and intercept of the curve. All arithmetic operations, including testing for significance by the *t*

* Some of these data have been published in abstract form (1).

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...ere carried out with a Hewlett-Packard programmable ...lator, model 9810A.

In Vitro Assay—The method of assay for erythropoietin activity *in vitro* is a semimicro modification derived from two papers published earlier (9, 10). A brief description of this technique, as yet unpublished, is presented here. Bone marrow cells from 10- to 12-week-old male rats, at a concentration of 15 million nucleated cells per ml, are suspended in a medium consisting of 65% NCTC 109 containing 30 mM morpholinopropanesulfonic acid and 0.05 mg per ml of gentamicin (Schering Corp.), at pH 7.0, 30% fetal calf serum, and 5% rat serum containing 17.7 μg per ml of ferric nitrate (11). Aliquots (0.2 ml) are pipetted into the wells of culture plates (Linbro Chemical Corp. model FB-16-24-TC); erythropoietin standards from 1.0 to 10.0 milliunits and test solutions are added to the appropriate wells, six replicates being used for each group. The plates are sealed, incubated at 37° for 20 hours, after which 20-μl aliquots of ^{59}Fe-labeled rat serum are added to each well, and incubation continued for 5 additional hours. At the end of the incubation the cells are washed over into culture tubes (13 × 100 mm) with 0.145 M NaCl, 0.013 M phosphate, pH 7.3, centrifuged in the cold, washed once with the same solution, and twice with 1 ml of cold 5% trichloroacetic acid. The pellet, after the second trichloroacetic acid wash, is suspended in 0.4 ml of the same phosphate-buffered NaCl solution, ...l of Drabkin's solution (12 mM $NaHCO_3$, 0.8 mM KCN, and ...M $K_3Fe(CN)_6$), and 0.05 ml of a carrier rat hemoglobin solution (2.5 mg per ml) are added. The tubes are then counted to determine the total iron uptake, after which 0.1 ml of 1 M HCl is added and the contents are mixed, then 2 ml of cyclohexanone are added and mixed again very thoroughly. The tubes are centrifuged to separate the phases and 1-ml aliquots of the upper (organic) phase removed to determine the radioactivity in hemin. Plots of ln milliunits in the standards *versus* ln counts per min in either total iron or hemin are generally linear, and unknown amounts are determined from the slope and intercept of the standard curve. When polycythemic rats were used as the source of marrow cells, they were made plethoric by the method previously described (12).

In the studies of stability of desialated erythropoietin, the *in vitro* assays were done with the use of graded amounts of asialoerythropoietin as standards when the modified hormone was used, and native erythropoietin as standards when the unmodified hormone was studied.

Preparations of erythropoietin were desialated either enzymically or chemically. The enzymes used were a protease-free sialidase derived from *Vibrio cholerae* (a gift of Dr. Saul Roseman, Department of Biology, The Johns Hopkins University) and an agarose-bound sialidase (Sigma Chemical Co.). In a test of the former enzyme by means of a "glycoprotein" substrate (Nutritional Biochemical Co., assayed at 18.3% sialic acid), we found that 1 μl of enzyme solution liberated 8.1 nmoles of sialic acid per min in 0.1 M acetate buffer, pH 4.5, containing 0.01 M $CaCl_2$ at 37°. For desialation of erythropoietin, we used conditions that would liberate 20 times more sialic acid than was present in the hormone solution, thus assuring complete desialation. The agarose-sialidase was also used in large excess (260 nmoles liberated per min for 10 min in 0.2 M acetate buffer at pH 5.0 and 37°).

Acid desialation was done with 0.05 M HCl at 80° for 30 min, causing removal of essentially all of the sialic acid.

Treatment with galactose oxidase (Kabi, kindly given to us by Dr. T. Steck) was as described by Morell *et al.* (4). Determination of free sialic acid was done by the method of Aminoff with the use of alkaline Ehrlich's reagent (13).

To study the stability of native and asialoerythropoietin toward heat, 10-μl samples of hormone, containing 330 milliunits, were put into glass tubes (2 × 10 mm), sealed, and placed into a boiling water bath for the indicated times, after which the tubes were placed in an ice bath. The chilled tubes were opened, and 6-μl aliquots were removed and diluted with 100 μl of 0.1% bovine serum albumin in 0.15 M NaCl. The resulting solutions, nominally ...ining 1.9 milliunits per μl, were then assayed *in vitro*.

...n the experiments with trypsin, the two forms of the hormone (6-μl aliquots of native and asialoerythropoietin containing 180 milliunits) were put into 0.3-ml Reactivials (Pierce Chemical Co.) with 0.3 μl of a trypsin (crystalline, Worthington Biochemicals, Inc.) solution containing 2 mg per ml in 0.145 M NaCl, 0.013 M phosphate, pH 7.3. After the indicated times at 23°, 0.3 μl of soybean trypsin inhibitor (1 mg per ml in the same buffered saline, Sigma Chemical Co. type 1-S) was added to each vial. The samples were then diluted with 100 μl of 0.1% bovine serum albumin in 0.15 M NaCl to yield a solution containing nominally 1.7 milliunits per μl. Aliquots (5 μl) were assayed *in vitro* with the use of either native or asialoerythropoietin as standards. The amount of soybean trypsin inhibitor required for complete inhibition of the amount of trypsin used was determined by the use of the synthetic substrate *N*-α-benzoyl-DL-arginine-*p*-nitrosoanilide-HCl. We also determined that the quantity of trypsin inhibitor complex plus excess inhibitor that was added to the marrow cell cultures had no effect on either the base-line or response to erythropoietin.

The erythropoietin used was a preparation derived from anemic sheep plasma, with a potency of 200 units per mg of protein. In one experiment (Fig. 3*B*), erythropoietin from the same source with a potency of 9000 units per mg of protein was used. This preparation represents essentially pure erythropoietin (14).

Human orosomucoid was prepared by the method of Whitehead and Sammons (15). Stachyose and β-galactosidase (*Escherichia coli* grade IV) were bought from Sigma Chemical Co. Asocoll was bought from Calbiochem.

TABLE I

Competitive effects of galactose-terminal compounds

Preparation	Units	Recovery	p[a]
		%	
Erythropoietin	8.1	100	
Asialoerythropoietin	0.0	0	
Asialoerythropoietin + asialoorosomucoid[b]	2.0	25	<0.02
Asialoerythropoietin + stachyose[c]	2.6	32	0.05
Asialoerythropoietin + lactose[c]	0.1	0	

[a] p = probability of the test group being the same as the control group (no erythropoietin), estimated by t test.

[b] Asialoorosomucoid given intravenously, 10 mg at −1, 0, and +1 hours with respect to erythropoietin injections.

[c] Stachyose given intravenously, 3 mg at −2, 0, and +2 hours with respect to erythropoietin injections. Lactose was administered in the same manner as stachyose.

RESULTS

The activity of asialoerythropoietin in rats pretreated with asialoorosomucoid, the tetrasaccharide, stachyose,[1] or lactose is shown in Table I. Whereas lactose had no effect, both the asialoglycoprotein and stachyose did permit 25 to 30% of the original erythropoietin activity to be manifest. On a molar basis, asialoorosomucoid is about 20 times more effective than stachyose.

The data in Table II show the effect on *in vivo* activity of asialoerythropoietin after enzymic galactose oxidation. Regardless of whether desialation was done enzymically or by acid, oxidation with galactose oxidase resulted in 40 to 50% restoration of the activity by *in vivo* assay. In contrast, oxidation with periodate with the use of the conditions described by Spiro (16), which should have caused the complete loss of only terminal galactose residues among the carbohydrates, did not restore any *in vivo* activity (Table II). Similarly, the use of β-galactosidase (shown to be free of any protease using Asocoll as substrate) for 24 hours did not result in any restoration of activity (Table III). No activity was found even when the enzyme had been allowed to act for 72 hours. The conditions of β-galactosidase treatment were chosen by measuring the rate of the enzymic

[1] We are indebted to Dr. Lennart Rodén for suggesting the use of stachyose.

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TABLE II

Effect of galactose oxidation on asialoerythropoietin activity in vivo

Preparation	Units	Recovery	p*
		%	
Erythropoietin	9.0	100	
Asialoerythropoietin (soluble sialidase)	0.0	0	
Asialoerythropoietin after galactose oxidase treatment	3.7	41	0.02
Erythropoietin	8.3	100	
Asialoerythropoietin (acid hydrolysed)	0.7	8	
Asialoerythropoietin after galactose oxidase treatment	4.1	49	<0.001
Asialoerythropoietin (soluble sialidase)	0	0	
Periodate-oxidized asialoerythropoietin	0	0	

* p = probability that the test group is the same as the control group (no erythropoietin), estimated by t test.

TABLE III

Effect of β-galactosidase on asialoerythropoietin

Desialation was done with agarose-bound sialidase; treatment with β-galactosidase (0.2 unit) was for 24 hours at 25° in 0.11 M phosphate, 0.05 M acetate, pH 6.9.

Preparation	Units	Recovery
		%
Erythropoietin	11.8	100
Asialoerythropoietin	0.2	1.7
Asialoerythropoietin after β-galactosidase	0.2	1.7

release of galactose from asialoorosomucoid. Using these conditions, 10 μg of enzyme caused the release of 1 nmole of galactose per min. All of the newly terminal galactose residues of asialoerythropoietin should have been hydrolysed in 100 min if it had been a similar substrate for the enzyme.

Because periodate oxidation did not restore any *in vivo* activity, we determined whether it had caused the loss of *in vitro* activity as well. The results (Table IV) show clearly that the conditions of periodate treatment, which were thought to affect terminal galactose residues predominantly, caused the loss of about 90% of the *in vitro* activity. This slight residual activity could not have been detected *in vivo*.

Since the restoration of *in vivo* activity by the use of galactose oxidase was not complete, we thought it of interest to study the relative stabilities of erythropoietin and asialoerythropoietin. In these experiments, the biological activities were determined by the *in vitro* method, and the same preparations, whether native or desialated, were used for the experimental points and the standard dose-response curves. As shown in Fig. 1, asialoerythropoietin is significantly less stable toward heat than the native hormone. The half-life of the native form at 100° is approximately 9 min, whereas that of the desialated form is about 4 min.

The desialated hormone is also more susceptible to tryptic hydrolysis than the native hormone (Fig. 2). We observed a lag of about 5 min before there was any appreciable fall in activity due to trypsin, after which the native hormone had a half-life of about 1.8 min, whereas the half-life of asialoerythropoietin was about 0.8 min. In both of these experiments total iron uptake was measured. Essentially the same results were obtained when heme synthesis was determined.

TABLE IV

*Effect of periodate on asialoerythropoietin assayed in vitro**

Preparation	Counts per min	Δ Counts per min
Control	1480 ± 170	
Erythropoietin		
1 milliunit	1860 ± 110	380
2 milliunits	2090 ± 170	610
4 milliunits	2510 ± 70	1030
Asialoerythropoietin		
2 milliunits	2660 ± 130	1280
4 milliunits	3220 ± 60	1740
Periodate treated asialoerythropoietin, 4 milliunits	1620 ± 90	140

* *In vitro* assay: only total iron uptake was measured.

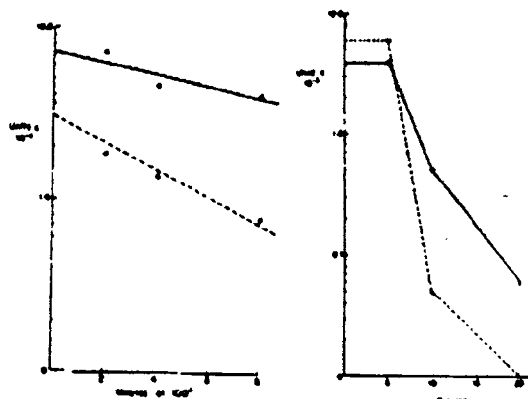

FIG. 1 (*left*). Heat inactivation of erythropoietin and asialoerythropoietin. Assays were done *in vitro* measuring total iron uptake. ●——●, erythropoietin; ○- - -○, asialoerythropoietin.

FIG. 2 (*right*). Trypsin inactivation of erythropoietin and asialoerythropoietin. Assays were done *in vitro* measuring total iron uptake. ●——●, erythropoietin; ○- - -○, asialoerythropoietin.

We have also found that asialoerythropoietin is significantly more active *in vitro* than the native hormone. At low dose levels the increase is about 3-fold. This is true whether the erythropoietin used is impure (200 units per mg of protein, Fig. 3A) or pure (9000 units per mg of protein, Fig. 3B). When desialation was done with the use of acid or agarose-bound sialidase, thus preventing any enzyme from being carried over to the cell suspension used for assay, a factor of 3 was still observed.

Since erythropoietin acts on several types of cells, including some that are already on the differentiated erythroid pathway (12, 17, 18), it was possible that asialoerythropoietin might be acting only on this latter cell type in the marrow population and that the increased activity would not be found with the more primitive target cells. A test of this by the use of bone marrow from artificially polycythemic rats, which is very much depleted with respect to differentiated erythroid cells but still contains the primitive erythropoietin responsive cells, showed that the in-

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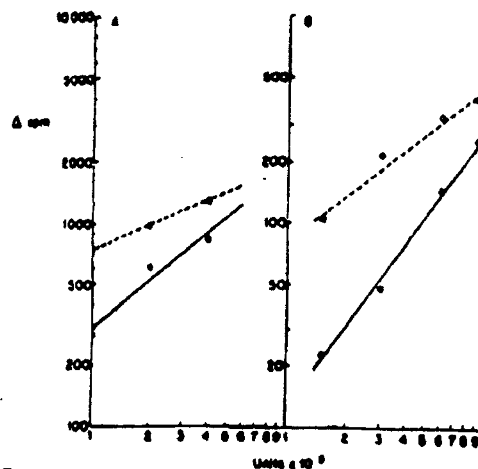

FIG. 3. *In vitro* dose-response curves for erythropoietin and asialoerythropoietin. *A*, erythropoietin potency was 200 units per mg of protein by *in vivo* assay. ●——●, erythropoietin; ○- - -○, asialoerythropoietin. *B*, erythropoietin potency was 8000 units per mg of protein by *in vivo* assay. ●——●, erythropoietin; ○- - -○, asialoerythropoietin in both *A* and *B* total iron uptake was measured.

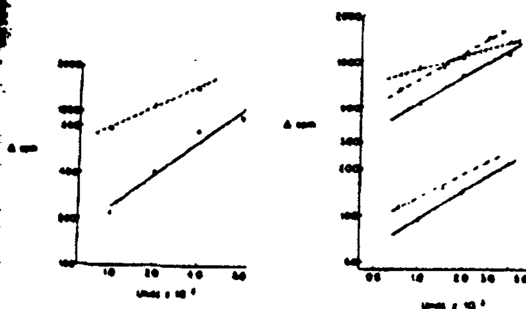

FIG. 4 (*left*). Effects of erythropoietin and asialoerythropoietin *in vitro* on marrow cells from plethoric rats. Total iron uptake was measured. ●——●, erythropoietin; ○- - -○, asialoerythropoietin.

FIG. 5 (*right*). Effects of modified erythropoietins *in vitro*. The *upper lines* represent total iron uptake, the *lower lines* represent heme synthesis. ●——●, erythropoietin; ○- - -○, asialoerythropoietin; Δ- - -Δ, galactose-oxidase treated asialoerythropoietin.

creased activity was still detected (Fig. 4). Asialoerythropoietin had about 5 times more activity than the native hormone at the lower end of the dose-response curve, and about 3 times more activity at the upper end. As with cells from normal rats, the slope of the ln dose-ln response curve for the desialated hormone was significantly lower than that for the native form.

In vitro assay of galactose oxidase-treated asialoerythropoietin in comparison with both the native and desialated hormones showed that, after enzymic oxidation, the slope of the dose-response curve was about the same as that for the unmodified hormone (0.58 and 0.56); there still was a significant increase in activity, however, in this case about 2-fold (Fig. 5).

DISCUSSION

A number of the findings reported here with erythropoietin are consistent with those reported by Morell *et al.* (5) for other glycoproteins. Thus, the competitive effects of asialoorosomucoid and stachyose that permit some activity *in vivo* with asialoerythropoietin and the effects of galactose oxidase on both *in vivo* and *in vitro* activities strongly suggest that the sugar exposed by removal of sialic acid is galactose. The preliminary estimate of the carbohydrate composition of erythropoietin (19) indicates that there is at least one galactose present per sialic acid residue.

Failure to restore activity *in vivo* by use of β-galactosidase may simply indicate that asialoerythropoietin is a much poorer substrate for the *E. coli* enzyme than either asialoorosomucoid, used in our preliminary experiments, or asialofetuin, used by Spiro (16). If the galactose were held by an α linkage rather than the usual β, the lack of action of β-galactosidase would be expected. Some precedent exists for the occurrence of α-galactosides in glycoproteins from red blood cells (20). Our observation that lactose is not an effective competitor confirms the finding of Morell *et al.* (5) and, since stachyose is competitive, indicates that the minimum effective size for an oligosaccharide terminating in galactose must be either 3 or 4 residues. There are a number of trisaccharides that could be tested in this regard; we have not done so.

Removal of sialic acid from erythropoietin results in increased lability toward heat and trypsin. These changes may be indicative of a conformational change and an accompanying general decrease in stability, and may explain why only part of the *in vivo* activity can be restored by oxidation of galactose. If there is a change in conformation accompanying desialation it does not appear to affect the interaction between human erythropoietin and an antibody toward it (21). Samy (22) has shown that a glycoprotein derived from sheep plasma was more susceptible to tryptic hydrolysis after desialation. This glycoprotein lost its ability to inhibit both trypsin activity and the clotting reaction upon removal of sialic acid.

Our data indicate that removal of sialic acid from erythropoietin causes an appreciable increase in its activity *in vitro*. Similar findings have been reported by Dufau *et al.* (23) who have shown that human luteinizing hormone and chorionic gonadotropin have increased affinity for testicular or ovarian receptors after desialation. Lukowsky and Painter (6) found a similar effect but ascribed it to the action of sialidase on the marrow cells. Since we found the increase in activity even when desialation was done by acid hydrolysis or by the use of agarose-bound sialidase, and since Dahlberg (24) has shown that treatment of marrow cells with sialidase does not cause any change in their response to erythropoietin, we conclude that the increase in activity is intrinsic to the asialohormone. This increase may reflect the fairly large reduction in negative charge that accompanies desialation. If the target cells are negatively charged, the presence of 16 to 18 strong anionic groups on the native hormone may retard interactions with the cells; the asialohormone might then have easier access to the cells. A similar situation obtains with human chorionic gonadotropin where the asialo form of the hormone has a higher affinity for receptor sites than the native hormone (25).

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