EXHIBIT C

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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being investigated, and the results will be reported at a later date.

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Inactivation of Erythropoietin by Neuraminidase and by Mild Substitution Reactions

ERYTHROPOIETIC activity in the plasma of rabbits made anaemic with phenylhydrazine is associated with mucoproteins[1-4]. It has been shown that the activity is destroyed by proteolytic enzymes[1,3,5]. Rambach *et al.*[4] have found as well as us that heating at 100°, *p*H 2, for 1 hr. destroys the activity, a treatment which, in the case of guinea pig α_1-acid glycoprotein[2], selectively removed sialic acid. In spite of the mildness of this treatment it is possible that other bonds (sugar, amino-acid) were broken. We have now been able to show that exposure to neuraminidase[6] completely inactivates the mucoprotein.

An acid glycoprotein (15·6 per cent sialic acid) has been regarded as erythropoietin by Rambach *et al.* We[2] have pointed out that it is probable that only a small proportion of the concentrates yet prepared is the active erythropoietic material. Recent work by Campbell *et al.*[11,12] with sheep plasma has strengthened this conclusion. Their most active preparation is lower in sialic acid.

The cold, neutralized filtrate from heat-coagulated anaemic rabbit plasma[3] is mixed with 1·5 vol. of absolute ethanol and allowed to stand at 5° overnight. The inactive precipitate is removed by centrifugation in the cold and the supernatant mixed with an equal volume of absolute ethanol. After standing overnight, the precipitate is collected in the cold and dried in a desiccator. It is extracted with 20 parts of 0·01 *M* sodium acetate, *p*H 4·8, and the soluble portion (about 85 per cent) is dialysed against distilled water. The dialysand is lyophilized. 60–120 mgm. of a very soluble white powder are obtained from 1 litre of plasma. We will refer to this preparation as *LD*.

When *LD* is applied to a column of 'DEAE' cellulose (1 gm.) equilibrated in 0·01 *M* sodium acetate, *p*H 4·8, 15–30 per cent of protein emerges as a front, 10–20 per cent as another peak with 0·05 *M* sodium chloride as eluant. Both are erythropoietically inactive. Elution with 0·1 *M* sodium chloride yields a peak containing all the activity and 50–60 per cent of the protein. It again gives a single peak when rechromatographed on a similar column. Its properties are listed in Table 1.

A potent human urinary ultrafiltration residue[3], a gift of Dr. Van Dyke, was applied to a 'DEAE' column as above. 0·25 *M* sodium chloride had to be used for elution of the activity. Paper electrophoresis at *p*H 8·6 and 4·5 showed it to be similar to *LD* (mobility like an acid α_1 protein). Similar behaviour is shown by erythropoietin obtained from unboiled sheep plasma before its final purification[11,12].

Table 1

	Buffer	*p*H	Ionic strength	Mobility × 10⁵ cm.² volt sec.⁻¹
Moving boundary electrophoresis.	Sodium barbiturate	8·75	0·1	− 5·40
	Sodium acetate	4·25	0·1	− 2·92
	Glycine hydrochloride	2·34	0·1	+ 0·70
Isoelectric point		2·75		
$E(1\ per\ cent,\ 1\ cm.)\ 280\ m\mu$		7·0		
Protein bound hexose (orcinol)		9·4%		
Hexosamine (Elson-Morgan, Rimington)		9·0%		
Sialic acid (resorcinol hydrochloride, Svennerholm (ref. 17) 15 min., 100° C.)		13–14%		
Methyl pentose		0·66–0·80%		

Neuraminidase treatment. (*a*) 15 mgm. *LD* in 2·4 ml. 0·05 *M* calcium acetate, *p*H 6, were incubated with about 30,000 units of neuraminidase[6] at 35°. After 1 hr., 0·1 ml. of 1 *M* sodium citrate and 10 ml. water were added, and the mixture boiled for 5 min. It was erythropoietically inactive. No loss of activity was found in a control experiment without enzyme added. (*b*) In similar experiments the reaction was stopped by sodium citrate alone without boiling after 10 min. and 1 hr. respectively, and each reaction mixture was then dialysed against 10 vol. of water. In both cases practically all the sialic acid of *LD* was found in the dialysate, and both dialysates and dialysands were erythropoietically inactive. Peptide bond hydrolysis (increase in amino-nitrogen) was insignificant and probably did not occur.

A number of relatively mild substitution reactions were carried out with *LD*:

(1) *Iodination* (ref. 13). To 10 mgm. *LD* in 0·4 ml. 0·35 *M* sodium phosphate *p*H 7·6, increments of 0·1 *N* iodine were added at room temperature. 0·07–0·13 ml. were taken up in 3½ hr.

(2) *Acetylation* (ref. 14). To 10 mgm. *LD* in 0·2 ml. of a solution of 11·9 gm. sodium acetate in 20 ml. water (*p*H = 9·25) 0·03 ml. of acetic anhydride was added over a period of 1 hr. at 0°.

(3) *Formaldehyde* (ref. 15). 10 mgm. *LD* in 0·1 ml. 0·35 *M* sodium phosphate *p*H 7·6 and 0·01 ml. 40 per cent formaldehyde were allowed to stand for 20 hr. at 8°. Excess formaldehyde removed *in vacuo*.

(4) *Esterification* (ref. 16). To 10 mgm. *LD* in 0·4 ml. methanol at −15° 0·02 ml. of thionylchloride were added over 3 hr. Methanol was removed *in vacuo*.

All four of the above reaction mixtures were erythropoietically inactive. (Assayed by iron-59 uptake in fasted rats[3,13].)

The enzymatic loss of erythropoietic activity indicates that bound sialic acid is an essential part of the molecule. It remains to be seen whether the high sensitivity of the molecule to substitution reactions carried out under conditions too mild for hydrolysis is related only to the sialic acid moiety or other sugar or amino-acid constituents of the active mucoprotein.

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Co-factors in *Hevea brasiliensis* Latex Serum

It has recently been shown[1] that the incorporation of mevalonic acid into rubber by an enzyme system in *Hevea* latex *in vitro* is not dependent on the addition of such co-factors as are known to be necessary for the incorporation of mevalonic acid into squalene. Such co-factors include adenosine triphosphate, cysteine and/or glutathione in the case of cell-free preparation of rat liver[2], although Amdur, Rilling and Bloch[3] found that whereas adenosine triphosphate was necessary with a soluble extract of bakers' yeast, no reducing agent requirement was demonstrated. Popjak and Gore show that under anaerobic conditions the reducing agent is not necessary but it is essential for the formation of squalene aerobically. Tchen[4] has demonstrated that the purified enzyme 'mevalonic acid-kinase' responsible for part of the pathway, namely, the phosphorylation of mevalonic acid, requires the presence of any of the four nucleoside triphosphates (including cytidine triphosphate). He shows also that the enzyme is inhibited by chloromercuribenzoate.

The present communication is to record the detection and assay of di- and tri-phosphates of adenosine and cytidine and of cysteine and reduced glutathione in samples of freeze-dried fresh latex sera obtained from Malaya. This material[5] would be expected to be equivalent to the serum from fresh latex 1–2 hr. after tapping, so far as soluble co-enzyme content is concerned. Details of these investigations will be published elsewhere; but it is thought of interest to record that such samples of normal latex sera examined contain approximately 0·7 micromole of glutathione and 0·4 micromole of cysteine per ml. and at least 0·0005 per cent nucleoside diphosphate in the form of adenosine diphosphate and cytidine diphosphate (predominantly as adenosine diphosphate), and almost the same amount of nucleoside triphosphate (predominantly as cytidine triphosphate).

It would be of interest to determine the influence of —SH inactivating agents on both the aerobic and anaerobic incubation of mevalonic acid labelled with carbon-14 in fresh latex to establish the similarity or otherwise of the general reductive mechanism in the two instances.

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Isolation of the Neurotoxic Component of the Venom of the Sea Snake, *Enhydrina schistosa*

THE sea snake, *Enhydrina schistosa*, is widely distributed in the coastal regions of the Far East and many fatalities from its bite are reported[1]. The *LD*50 of the venom for a wide variety of laboratory mammals is in the region of 50–100 μgm./kgm. body-weight. On this basis it is four times as toxic as the venom of cobra. Venom yields per bite of up to 55 mgm. of dried venom have been obtained from captive snakes. If humans are of equal susceptibility to laboratory animals, up to 15 *LD*50 for humans may be expected from one bite. At present there is no supply of antivenom available for treatment, and it is doubtful if the titres obtainable by even the best available preparative methods would be sufficient to allow the antivenom being administered in a life-saving dose. The separation of the neurotoxic component of this venom is important from the point of view of analysing its biochemical actions and enhancing its antigenic properties.

Carey and Wright[2] have shown that the whole venom consists of at least three antigenic components. The neurotoxic component is the most electropositive (*p*H 6·8 in agar gel) and will pass through a cellulose but not a nylon dialysis sac.

In the present investigation it has been found that after repeating the cellulose sac dialysis against large volumes of distilled water approximately 1 per cent of the initial toxicity remains inside the sac. This may be due to a different toxic component or to adsorption of traces of dialysable component on to non-dialysable material. However, it is unlikely to contribute significantly to the toxicity of the whole venom. Immunoelectrophoresis of the cellulose sac dialysate using antiserum to the whole venom shows only one main precipitation line. However, this has since been shown to consist of at least two antigenic components by varying the relative concentrations of venom and antiserum in the immunoelectrophoresis. When the dialysate was placed on a carboxy methyl cellulose column and eluted with *p*H 6·6 buffers of graded molarities from 0·06 to