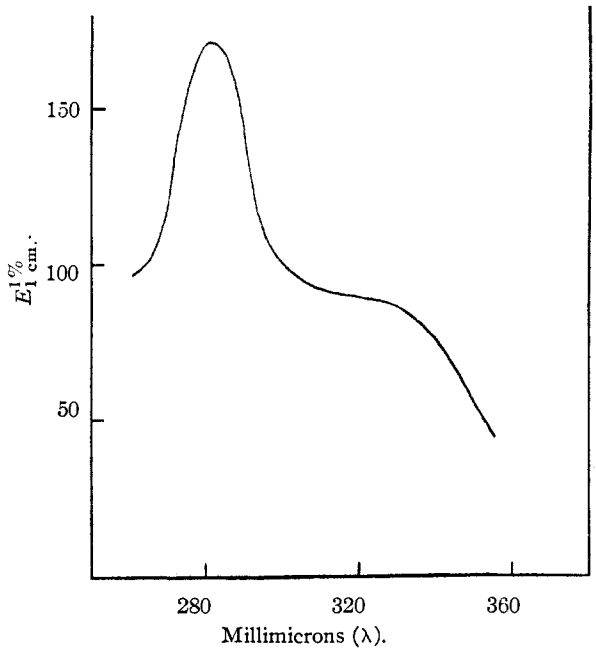

Fig. 2.—Spectrum of kiri native lignin.

It is readily observed that the methoxyl content of each of the chemical lignins is higher than that of the native lignin fraction. This discrepancy is due to either a change in the lignin complex on extraction with strong chemical reagents,[6] or to the fact that the total lignin present in kiri wood is inhomogeneous. Since kiri wood gave a strong positive Mäule color test,[7] which is specific for the presence of syringyl groups,[8] whereas the native lignin fraction gave only a fading cerise color, it appears that the latter explanation is more nearly correct. A similar situation was encountered in the study of the native lignins from oak and birch.

Experimental

Isolation of Native Lignin.—Air-dried kiri wood was stripped of its bark, ground to 40 mesh, and extracted with water and with ether. It was then extracted with ethyl alcohol until it no longer gave the phloroglucinol–hydrochloric acid color test. The alcohol was removed at reduced pressure. The reddish-brown residue was again washed with water and ether and dried *in vacuo*. It was then dissolved in dioxane, centrifuged, filtered and precipitated into an excess of ice-cold distilled water. The precipitate was dried, redissolved in dioxane to make a 10% solution and precipitated into ether. The above procedure was repeated until a constant methoxyl value was obtained.

The acetate and phenylhydrazone derivatives were prepared by the usual methods.[8]

Isolation of Chemically Prepared Kiri Lignins.—Extractive-free kiri wood was used in all cases; *i.e.*, the ground material was extracted in a Soxhlet apparatus first with 1:2 alcohol–benzene solution, and then with water.

Sulfuric acid lignin was isolated by the standard method.[9] The lignin content of the wood was found to be 22%, on a moisture-free basis. Alkali lignin[10] was also isolated by a standard method.

Ultraviolet Absorption Spectrum.—A solution of the lignin sample was prepared for spectroscopic analysis by dissolving 1 to 2 mg. of the sample in 50 ml. of the solvent (90 parts of purified dioxane to 10 parts of distilled water). A Beckman quartz spectrophotometer was used for the determination of the absorption curve.

Infrared Absorption Spectrum.—A Perkin–Elmer double beam recording infrared spectrophotometer was employed in this study.

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Preparation and Properties of Serum and Plasma Proteins. XXXIII. Specific Interactions of Prothrombin and Other Proteins with Barium Sulfate

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The effectiveness of barium sulfate in rendering plasma incoagulable, first observed by Bordet and Delange in 1912,[2] renders convenient the purification of prothrombin and serum prothrombin conversion accelerator and its plasma precursor. Alexander has demonstrated[3] that citrate brings about resolution of these proteins from barium sulfate, a finding which explains why prothrombin is not removed from citrated plasma by barium sulfate.

Calcium-free plasma, obtained by passage of blood over an ion exchange resin, yields to barium sulfate a fraction comprising 1% or less of the plasma proteins. This fraction contained, in addition to prothrombin and the precursor of serum prothrombin conversion accelerator, certain other proteins, apparently not involved in the coagulation mechanism. Sodium acetate, sodium chloride, glycine and glucose had no effect on the interaction; oxalate, which did not inhibit the interaction of prothrombin with barium sulfate, diminished the amount of inert protein removed.[4]

In our studies on the purification of prothrombin, we have investigated the effect of a number of substituted carboxylic acids on the interaction of these proteins, particularly of prothrombin, with barium sulfate.

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Experimental

Human plasma, from blood collected by passage over a cation exchange resin (Dowex-50) on the sodium cycle to remove calcium, was treated, in 10-ml. aliquots, with barium sulfate (C.P. Baker), 0.1 g./ml. of plasma, in centrifuge tubes.[5] All operations were carried out at room temperature. After stirring with a glass rod for 10 minutes, the barium sulfate was separated by centrifugation. The precipitate was then resuspended, stirred for 10 minutes, and recentrifuged, using 10-ml. quantities of the following reagents in sequence: (a) 0.15 *M* sodium chloride, (b) 0.1 *M* solution of the sodium salt of a substituted carboxylic acid, adjusted to *p*H 7.4–7.6, (c) 0.15 *M* sodium chloride, and (d) 0.1 *M* sodium citrate. Solutions (a) and (c) did not contain prothrombin and were discarded. Solutions (b) and (d) were analyzed for nitrogen and prothrombin content.

Assay for prothrombin was carried out by the two-stage procedure using Armour Bovine Fibrinogen (Fraction I) as substrate.[6] Nitrogen was determined by nesslerization of the digested samples. The total yield of prothrombin in extracts (b) plus (d) was essentially quantitative in every experiment. We are grateful to Dr. B. Alexander for the prothrombin analysis.

Results

The data are summarized in Table I. The acids tested have been arranged in order of their association constants for barium in the reaction $M + X \leftrightarrows MX$, where M is the metal ion and X the ligand. In the case of compounds for which the barium association constant was unknown, comparison with the constants of α-hydroxy acids for barium or with the known constants for calcium or magnesium have been tentatively relied upon.

The results suggest that, analogous to the behavior of other insoluble compounds of the alkaline earth metals, the interaction with barium sulfate consists in the formation of a metal–protein complex. Further, cation exchange resins on the barium cycle, both of the sulfonated type (Dowex-50) and the carboxylated type (IRC-50) have been found effective in removing prothrombin from calcium-free plasma.

Half of the proteins interacting with barium sulfate other than prothrombin, were extractable therefrom by compounds of known affinity for barium. The amount extracted was related to the association constant. The results in Table I suggest a high order of specificity for the interaction with barium.[7] Oxalate and diglycolate, with association constants near that of citrate, were ineffective in removing prothrombin from barium sulfate. The ineffectiveness of tricarballylate and aconitate, lacking the β-hydroxyl group, but with lower affinities for barium, suggests that a steric requirement may operate in addition to the affinity for barium. The partial effectiveness of ethylenediaminetetraacetate and its cyclohexyl derivative suggests that their steric dissimilarities are overcome, at least in part, by their high affinities for barium, approximately 10,000 times that of citrate. Another compound with a known affinity for barium, the sulfate ion, while ineffective at 0.1 *M*, extracted prothrombin at 0.5 *M* (as sodium sulfate). Sodium chloride failed to extract prothrombin at concentrations up to 2 *M*. Addition of barium ions to the plasma resulted in decreased interaction of prothrombin with barium sulfate. Even at a concentration as high as 0.04 molar barium chloride, however, 70% of the prothrombin was adsorbed by barium sulfate; formation of the insoluble interaction product occurred in preference to reaction in solution.

Quick[8] in a study of the inhibition of the interaction of prothrombin with calcium phosphate by sodium citrate has postulated the formation of a complex between citrate and prothrombin which prevents adsorption. The present data suggest as a more probable alternative, competition between prothrombin and citrate for the metal.

The precursor of serum prothrombin conversion accelerator has not been separated from prothrombin under any of the above conditions. The active form, obtained by treating serum with barium sulfate,[9] behaved in the same manner, suggesting that the sites of binding on prothrombin and the serum prothrombin conversion accelerator are of similar nature.

TABLE I

THE EFFECT OF CERTAIN ANIONS ON THE DISSOCIATION OF PROTHROMBIN AND OTHER PROTEINS FROM THEIR STATE OF INTERACTION WITH BARIUM SULFATE

Nature of anion of 0.1 molar sodium salt	Affinity constants log *K* (Ba)	Nitrogen recovered (mg./ml.)[a]	Prothrombin recovered (units/ml.)
Acetate	0.39[b]	0.002	0
Lactate	0.55[b]	.002	0
α-Hydroxybutyrate	(0.55)	.002	0
Glycinate			0
Glutamate		(.04)	0
Aspartate	1.1[d]	(.06)	0
Succinate	1.21[d]	.023	0
Itaconate		.033	0
Malonate	1.23[b]	.041	0
Malate	1.26[d]	.062	0
Aconitate		.073	0
Diglycolate		.076	0
Tricarballylate	1.4[d]	.073	0
Tartrate	1.67[d]	.067	0
Oxalate	2.33[c]	.065	0
Citrate	2.54[d]	.128	115
Ethylenediaminetetraacetate	7.76[e]	(.07)	58
1,2-Cyclohexyldiaminetetraacetate			73

[a] The values in parentheses were corrected for non-protein nitrogen, and are therefore less reliable. [b] R. K. Cannan and A. Kibrick, THIS JOURNAL **60**, 2314 (1938). [c] R. W. Money and C. W. Davies, *Trans. Faraday Soc.*, **28**, 609 (1932). [d] D. Schubert, personal communication. [e] G. Schwarzenbach and H. Ackermann, *Helv. Chim. Acta*, **30**, 1798 (1947).

(5) Recently it has been found by Dr. Alexander that certain preparations of C.P. barium sulfate are more effective than others. With the best preparations, the amount required for complete removal of prothrombin can be reduced to as little as 0.025 g./ml. of plasma (B. A. Alexander, personal communication).

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