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The Reaction of Heparin with Proteins and Complex Bases

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Owing to its strongly acid nature, heparin forms stable salts with many proteins, examples of which have been reported by a number of workers, e.g. Reiner & Kopp [1928], Fischer [1931], Chargaff & Olsen [1937], and Scott & Fisher [1942]. The general nature of the reaction was first indicated by Fischer [1935] and Fischer & Astrup [1935] who studied its kinetics. While other examples of the reaction are now being reported, many authors appear to have overlooked these important contributions by Fischer, and in particular his finding that the reaction is a simple salt formation in which reversible dissociation of the salt occurs according to the mass law [Fischer & Astrup, 1935]. This latter point means that data may be analysed more thoroughly than hitherto, and that confusing results have frequently a simple physico-chemical explanation. During studies on heparin, the author has frequently confirmed Fischer's original observations in experiments carried out as controls for other studies. These findings, and the results of other experiments, are reported in this communication.

Three principles are available for the demonstration of the reaction of heparin with proteins. These are: (1) direct observation of the formation of the compound, e.g. precipitation of the gelatin-heparin compound when the two solutions are mixed; (2) the alteration in the properties of the protein, e.g. the shift in the isoelectric point (I.E.P.) of casein; the decrease in precipitability of serum globulin by alcohol, heat, and other precipitating agents [Fischer, 1935]; loss of activity by fumarase [Fischer & Herrmann, 1937]; (3) alteration in the properties of heparin, e.g. loss of dialysing power with egg albumin [Fischer, 1935] and loss of anticoagulant activity with protamines [e.g. Fischer, 1935; Chargaff & Olsen, 1937]. In our experience, it is necessary to choose a suitable method for each protein.

Failure to observe compound formation does not necessarily indicate that it has not occurred. Thus

when the heparin-protein compound is soluble under the conditions of the experiment (e.g. protamine in the presence of plasma) precipitation of the compound cannot be observed. The reaction can, however, still be detected by suitable indirect means (in this case by the loss of anticoagulant activity of the heparin).

Fischer indicated that a stoichiometric relationship existed between heparin and thromboplastin (and that presumably this extended to other proteins). Fischer & Astrup [1935] investigated this stoichiometric relationship more fully and found that the reaction could be represented as

$$\text{From the mass law, } \frac{C_{\text{protein}} \times C_{\text{heparin}}}{C_{\text{protein-heparin}}} = K.$$

Then, if a be the concentration of protein added, c the concentration of free protein, and b the concentration of added heparin, $a - c$ is the concentration of the product formed by the reaction, $b - (a - c)$ is the concentration of free heparin, and

$$K = \frac{c \{b - (a - c)\}}{a - c}. \quad (1)$$

Fischer & Astrup [1935] obtained satisfactory agreement with this equation for the mutual interaction of thromboplastin and heparin on hen plasma. The author has also found this to give good agreement for the reaction of heparin with casein, protamine, and toluidine blue, a non-protein basic substance. On the basis of these and other experiments, it seems likely that it is true of the reaction with all proteins, sufficient reversible dissociation taking place to have a significant effect on the kinetics of the reaction. The data of other authors show no disagreement with this view and tend to corroborate the general nature of the reaction. Actually, the earliest studies on heparin indicated that the biological action of heparin depended on such a reaction, since they showed that peptone

plasma could be clotted by simple dilution, i.e. by increasing the dissociation of the heparin-protein compound its anticoagulant effect could be annulled [Morawitz, 1905].

METHODS

Heparin was supplied by the Connaught Laboratories, University of Toronto, and had been crystallized as the barium salt. The sodium salt has also been used. Heparin was assayed either by the Howell cat method [Jaques & Charles, 1941], or by means of its metachromatic reaction with toluidine blue, when the Lovibond tintometer was used. The temperature for all experiments was 25°. I am indebted to Dr A. M. Wynne of the Department of Biochemistry for the use of an Evelyn photoelectric colorimeter.

Reaction of heparin with casein. This reaction is easily demonstrated, because although casein has an I.E.P. at pH 5.0, that of the casein-heparin compound is at pH 3.6. The compound is soluble at pH 5.0, and Fischer & Schmitz [1932] used this fact in assaying heparin. The I.E.P. of the casein was in the region of pH 4.6-4.9 (found value pH 4.8), whereas that of the casein-heparin was in the region of pH 3.5-4.3 (Table 1). The shift is as marked as that with eosin [Jordan-Lloyd & Shore, 1938]. The reaction was then studied at pH 5.0 by adding 1 ml. casein solution (50 mg./100 ml. N/10 Na acetate) to 9 ml. of N/180 acetic acid containing varying amounts of heparin and measuring the turbidity in an Evelyn colorimeter (Table 2). The turbidity values were converted to mg. of free casein by the experimentally determined equation $C = 3.96T + 0.0261$. Owing to the small portion of the scale used for the determination of turbidity, a considerable spread in the experimental values was found. The calculated values of K are given in Table 2, and show fairly good agreement. For convenience these values were calculated in terms of units of heparin and mg. of casein.

Reaction of heparin with toluidine blue. This important reaction was reported by Jorpes [1939] for use as a histological test for heparin. It consists in measuring the red colour of the compound. The reaction was studied with the aid of the Lovibond

tintometer. A sample of Groebler's toluidine blue was used, 1 ml. being added to 4 ml. of heparin solution in saline containing 0.25% Ca chloride. Table 2 shows that this compound formation is of the same type as that produced with proteins, the results showing good agreement with the mass law.

Table 2. *The reaction of heparin with casein and toluidine blue*

<i>a</i> (amount of casein added) = 0.5 mg. pH = 5.0			
Heparin added mg. $\times 10^{-2}$ = <i>b</i>	Turbidity	Free casein mg. = <i>c</i>	$K = \frac{c\{b - (a - \frac{1}{2}c)\}}{a - c}$
0.00	0.1206	0.503	—
0.153	0.1065	0.447	0.84
0.459	0.0757	0.326	0.53
0.765	0.0655	0.285	0.73
1.22	0.0518	0.269	0.82
1.53	0.0339	0.160	0.56
1.53	0.0458	0.208	0.88
4.59	0.0155	0.270	0.88
7.65	0.0044	0.0435	0.69
15.3	0.0000	0.000	—
Average			0.72

<i>a</i> (amount of toluidine blue added) = 5 mg./100 ml. pH = 5.0			
Heparin mg. $\times 10^{-2}$	Tintometer red reading	Combined dye mg./100 ml. = <i>a - c</i>	<i>K</i>
2.0	0.5	1.5	1.00
4.0	3.5	2.6	1.54
5.0	4.8	3.1	1.81
6.0	6.0	3.5	1.65
7.0	6.5	3.7	1.75
8.0	6.5	3.7	1.64
Average			1.56

Table 1. *Effect of heparin on the isoelectric point of casein*

- no precipitation. + degree of precipitation.			
pH	Casein + Heparin	pH	Casein + Heparin
5.80	± -	4.34	++ ++
5.31	+ -	4.14	++ ++
4.88	++ +	3.52	- ++
4.78	+++ ±	1.90	- ±
4.56	++ ++		

Casein (B.D.H. light white soluble), 1 mg./ml. Heparin, 0.1 mg./ml. Buffer, 0.2N K acetate-acetic acid. pH measurements by the Beckmann glass electrode.

Reaction of heparin with gelatin. While the reaction with casein is readily detected by the markedly different I.E.P.'s of the protein and of its compound with heparin, the reaction with gelatin, and also that with protamine, can be studied over a wider pH range, since the compounds formed between these proteins and heparin were found to be relatively insoluble over the range pH 2.5-10.5. Increasing amounts of gelatin were added to 2.4 mg. of heparin. The mixtures were centrifuged and the N content of the precipitates determined by the micro-Kjeldahl method. The heparin present in the supernatant was determined colorimetrically, that in the precipitate being calculated by difference. It can be seen (Table 3) that increasing the concentration of gelatin first increases, and then decreases, the amount of precipitate, i.e. the precipitate is soluble in the presence of an excess of gelatin. The composition of the precipitate, however, remains unchanged. It should be pointed out that not all samples of gelatin form an insoluble heparin compound. For these experiments the gelatin used was

an ordinary commercial gelatin, but a sample of Eastman Kodak technical gelatin, and also a sample of carefully prepared fish collagen (courtesy of Prof. N. B. Taylor) failed to give any precipitate with heparin over a wide range of concentration and pH.

Reaction of heparin with protamine. Chargaff & Olsen [1937] observed that when heparin and protamine are mixed an insoluble complex is formed, and showed by direct analysis of the complex that a simple salt formation had occurred. We mixed the protamine, salmine (Connaught Laboratories) and heparin in a total volume of 5 ml. containing 0.5 ml. of 10% CaCl_2 , the amount of precipitate formed being estimated by determining its N content by the micro-Kjeldahl procedure (Table 3). The values obtained for the dissociation constant showed that with this reaction also, partial dissociation of the compound occurs.

Table 3. *Reaction of heparin with gelatin and protamine (salmine)*

1 ml. of heparin (2.4 mg.) added to 3 ml. of gelatin solution. pH=3.9.

Gelatin N added mg.	N in ppt. mg.	Heparin in ppt. mg.	N/heparin in ppt.
0.66	0.050	0.26	—
1.31	0.714	1.53	0.47
2.62	1.41	2.06	0.68
4.58	0.798	1.85	0.43
6.55	0.606	0.97	0.62
13.1	0.122	0.26	0.48
26.2	No precipitate		

Heparin added to the protamine, salmine (Connaught Laboratories). Total volume=5 ml. pH=2.5. CaCl_2 concentration=1%.

Heparin mg.	Protamine mg. N	N in ppt. mg.	K
0.1	0.347	0.050	0.297
0.2	0.347	0.087	0.338
0.5	0.347	0.150	0.398
1.0	0.347	0.244	0.319
1.0	0.173	0.174	—
1.0	0.069	0.066	—
1.0	0.034	0.030	—
Average			0.338

Inactivation of heparin by proteins. As Fischer [1935] first indicated, certain proteins possess the power, not only of combining with heparin, but also of inactivating this anticoagulant. Chargaff & Olsen [1937] suggested the use of this reaction to inactivate heparin both *in vitro* and *in vivo*. The proteins possessing this property in the highest degree are the protamines, salmine and clupein. The protamine was thought to inactivate heparin by precipitating the latter, as in the above studies. If, however, protamine and heparin are mixed in the presence of plasma and in the concentrations normally used, precipitation does not occur, although inactivation

has taken place. That this is so can be seen by testing for the anticoagulant activity of heparin. This shows that protamine has combined with heparin to form an inactive compound.

The reaction can be further studied by adding increasing amounts of protamine to heparinized blood and determining the clotting time. As reported by Jaques, Charles & Best [1938] the clotting time decreases until it reaches the normal value found in the absence of heparin, and then increases again. In the presence of excess heparin or protamine the clotting time is prolonged and it is only at the equivalence point that the normal clotting time obtains. At the equivalence point the ratio of heparin to protamine was constant. This is true of any one protamine preparation, but more recent work has indicated that it is different for different preparations. Such a protamine titration is reported in Table 4. The experiment was carried out as previously described [Jaques *et al.* 1938], using the protamine, salmine, and adding 1 ml. of freshly drawn dog's blood to various mixtures of heparin and protamine. Also reported are the clotting times with heparin, and protamine alone. It can be seen that 0.5 mg. of heparin required 1.62 mg. of protamine to restore the clotting time to normal, i.e. 3.2 mg. protamine/mg. heparin. The same equivalence value was obtained with 0.1 mg. of heparin. It was shown that the reaction between protamine and heparin could be expressed by equation (1). The data indicate that in the presence of serum also dissociation of the protamine-heparin compound occurs. Thus the equivalence value for 0.5 mg. of heparin is 1.62 mg. protamine, whence 1.37 mg. protamine would combine with 0.42 mg. heparin. Thus there would be 0.08 mg. free heparin which would give a clotting time of over 24 hr. (0.008 mg. heparin gave a clotting time of 2 hr.). However, the clotting time with 1.37 mg. protamine was only 9 min. (corresponding to 0.002 mg.). The reason for this follows from the previous discussion, i.e. the large amount of protamine at the equivalence point is not that required to combine with heparin, but that required to suppress dissociation of the protamine-heparin compound. The values of the mass law dissociation constant calculated for the clotting times obtained with 0.5 mg. heparin were 0.39, 0.33, 0.44×10^{-2} (average = 0.39×10^{-2}). The average value of K for a sample of thymus histone, calculated from the data reported by Jaques *et al.* [1938] was 0.76×10^{-2} . Clearly, this dissociation is responsible for the sharpness of the titration of heparin with protamine. Protamine is only a weak anticoagulant, but such a large excess of the protein is required to suppress completely the dissociation of the compound that the concentration of protamine need be increased only slightly for it to affect the clotting time.

Fischer [1935] reported that a number of proteins possess the power to inactivate heparin. These were chiefly basic proteins, such as the protamines, histones and haemoglobin. Inactivation also occurs with other proteins, such as the liver proteins. In this case it is necessary to incubate the protein and heparin at an acid pH. Denaturation of the liver protein by heating results in the inactivation of more heparin. We have confirmed Fischer's results, using a saline extract of dog liver. Such experiments require careful controls, since the acid protein solution may lower the pH of the test blood thus making it less sensitive to heparin [Fischer, 1935], and suggesting that inactivation of the latter has occurred. Thus failure of the protein to annul the anticoagulant activity of heparin does not indicate that compound formation at the pH of the blood does not occur. For example, a gelatin-heparin mixture, in which almost all the heparin was found to be combined with the gelatin when the precipitate was removed and analysed, still showed full biological activity.

Table 4. *The effect of heparin and protamine on clotting time*

Heparin mg.	Clotting time (min.)	Protamine mg.	Clotting time (min.)
—	3	0.017	3
—	2	0.025	4
0.002	9	0.05	5
0.003	22	0.10	7
0.004	27	0.25	5
0.006	54	0.25	8
0.008	113	1.00	70

Protamine (mg.) added to 0.1 mg. heparin	Clotting time (min.)	Protamine (mg.) added to 0.5 mg. heparin	Clotting time
0.25	5	1.00	2 hr.
0.30	4	1.25	15 min.
0.32	2	1.37	9 min.
0.35	3	1.50	11 min.
0.40	6	1.62	2 min.
0.75	10	2.00	1 hr.

Factors influencing the dissociation constant of the heparin-protein compound. The dissociation of these compounds is markedly affected by the pH and the presence of other anions and cations (Table 5 and Fig. 1). These results have been obtained chiefly with toluidine blue, since it is relatively simple to obtain quantitative data with this base. Results obtained with a number of different proteins, however, indicate that the same conclusions apply to them. A change in pH from 4.1 to 6.2 (Table 5) resulted in a tenfold increase in the dissociation constant. Hence, as with salts of many organic bases, the corresponding heparin compound may often be completely hydrolysed by warming with dilute alkali, the free base being precipitated. The

presence of various cations and anions also results in increased dissociation of these compounds since they compete for the heparin and the base respectively. The presence of 0.5% CaCl_2 (Table 5) increased the dissociation constant fivefold. Since the equilibrium depends on the relative dissociation constants of the protein (or dye)-heparin compound and of the salts of protein and heparin with the inorganic anion and cation respectively, further increase in the concentration of the inorganic salt results in the removal of a large proportion of the base from the heparin (Fig. 1). Of the three salts shown, NaCl is as effective as CaCl_2 , suggesting that the effect is not due to the cation alone. The presence of proteins such as protamine or those of horse plasma similarly decreases the amount of heparin bound to the dye. Sufficient citrated horse plasma was included in one series to give a final concentration of plasma of 60% by vol. and in another series protamine (2 mg./100 ml.) was added. In each case less heparin combined with the dye. The protein is here acting as a cation, and it is interesting that even the weak plasma protein can act in this manner if sufficiently concentrated. The presence of a strong acid such as CCl_3COOH also results in the dissociation of the compound (Fig. 1). 5% CCl_3COOH completely inhibited the formation of the heparin-dye compound, even when a very large excess of heparin was present. 1% CCl_3COOH decreased the amount of heparin bound to dye by 50% when 0.05 mg. heparin was present.

Impurities in crude heparin preparations may exhibit the type of competitive inhibition shown in Fig. 1 to a marked degree. Thus, during assays by the dye reaction, it was found that the preparation showed no activity in concentrated solution. On diluting the preparation, however, heparin activity could be demonstrated. The effect of NaCl on the antithrombin activity of heparin, similar to that shown in Fig. 1 for its reaction with toluidine blue, has been reported previously [Jakues & Mustard, 1940]. In contrast to these results, Fischer reported that the presence of lipid increased the binding power of proteins for heparin.

Table 5. *Effect of changes of pH and of the presence of calcium chloride on the dissociation constant of the heparin-base compound*

Base	pH	K
Thymus histone	2.5	0.038
	4.1	0.043
	6.2	0.288
	8.2	1.68
CaCl_2 (%)		
Toluidine blue	0.0	0.60
	0.1	1.16
	0.2	1.56
	0.5	3.06

Fig. 1. Effect of various factors on the reaction of heparin with toluidine blue. pH 9.8 (borate buffer). Final concentration of reagents, dye, 5 mg./100 ml.; ZnSO₄, CaCl₂, NaCl, 5%; plasma, 60% (=4% protein); protamine, 2 mg./100 ml. CCl₃.COOH neutralized with NaOH.

DISCUSSION

The proteins here used were selected to illustrate certain extensions of Fischer's theory. As Fischer [1935] established, the reaction is a simple salt formation between the acid heparin and the basic protein. As such, it obeys the mass law so long as the product remains in solution. When, however, precipitation of the product occurs, there may be considerable deviations from the mass law. This is to be expected since these substances possess a high electrostatic charge and the calculations are necessarily based on concentrations and not on activities. Since heparin possesses two types of acid groups and there are differences between the various amino-groups of a protein, there is also the possibility that combination of heparin and protein may occur in more than one proportion, i.e. a series of compounds of the general formula P_xH_y , (P =protein, H =heparin) may be obtained. Heidelberger & Kendal have discussed this type of reaction with respect to the precipitin reaction. As reported above, the reaction between heparin and certain samples of gelatin shows an equivalence point and an inhibition zone, as in the antigen-antibody reaction. Since, however, the composition of the precipitate does not change under these conditions, the inhi-

bition phenomenon is probably best explained in terms of the electrostatic theory of electrolytes than by complex compound formation.

Fischer stated that combination between heparin and protein occurs only near the i.e.p., and on its acid side. With histone and gelatin however, this appears not to be the case. Although a shift in pH towards the alkaline side increases the dissociation of the compound, it is possible for heparin to combine with proteins far on the alkaline side of their i.e.p., e.g. gelatin precipitates with heparin even at pH 10. Hence, the greater part of the heparin in plasma must be bound to protein and not in the free state as suggested by Fischer. Further, it is possible by using a strong acid such as CCl₃.COOH to bring about dissociation of the heparin-protein compound even on the acid side of the i.e.p.

Two values are important in a quantitative study of the reaction between heparin and a given protein. One is the amount of protein which combines with 1 mg. of heparin, the other is the dissociation constant of the compound so formed. The former is best determined by preparing and analysing the compound, although Fischer & Astrup [1935] have suggested a possible graphical method. Since the molecular weight of heparin is not known, it is impossible to calculate the true dissociation con-

stant for even the simplest of these compounds. From equation (1), a function related to the dissociation constant can be obtained, but the concentration of the base must be expressed in terms of the equivalent amount of heparin, if it is desired to compare these values for different proteins. This has not been done in the case of the values so far reported, the constant having been calculated directly from the experimentally determined values in each instance.

The amount of protein which neutralizes the anticoagulant activity of 1 mg. of heparin is also of practical interest. This depends both on the amount of protein required to combine with the heparin and on the dissociation constant of the compound. Thus 4.1 mg. of toluidine blue combined with 1 mg. of heparin, but 7.1 mg. of the dye was required to neutralize its anticoagulant activity. The reaction represents a kinetic equilibrium, and if a second protein (or other cation) be added to a solution of heparin + protein the heparin will distribute itself between the two proteins, according to their relative concentrations and to the dissociation constants of the corresponding heparin compounds. Because the dissociation constant of the gelatin-heparin compound is greater than that of the compounds formed by heparin and the proteins of the clotting system in blood, if it is added to blood, practically all the heparin is transferred to the clotting system and its anticoagulant effect becomes apparent. If it is replaced by the protamine-heparin compound, however, no anticoagulant effect is observed, because the dissociation constant of the latter is smaller than those of the compounds formed by heparin with blood proteins.

Our general conclusions should be applicable to the reaction of heparin with any protein with which it forms such compounds, e.g. silk peptone, gelatin, clupein, salmine, histones prepared from thymus and blood, haemoglobin, egg albumin, casein, serum proteins, liver proteins, coagulant tissue extracts and thromboplastic protein, fumarase and trypsin. Reaction has also been shown to occur with a number of organic bases, namely, benzidine, quinine, brucine, piperidine, and the basic dyes, thionin and toluidine blue. When the protein involved exhibits enzymic or other biological activity the heparin will have an inhibitory effect, e.g. it has a slight inhibitory action on complement. Since the heparin salts of proteins are often entirely different from their parent proteins, showing different solubilities, i.e.p.'s, etc. (the criteria usually employed to classify proteins), the question arises as to whether heparin also affects the antigenic properties of such proteins. Kyes & Strauser [1926] reported that heparin inhibited anaphylactic shock in pigeons sensitized to sheep serum, and this was decisively confirmed by Van de Carr & Williams [1928], who used the sensi-

tized guinea-pig uterine strip (Dale). A number of other workers, including the author, have failed to confirm these findings. It is clear, however, from the previous discussion on the reaction between heparin and proteins, that such inhibitory action will depend to a high degree on the relative concentrations of heparin and protein, on the nature of the protein (including the degree of sensitization) and on the other proteins present. Hence it may be difficult to reproduce the necessary conditions.

Fischer & Astrup established that this type of reaction occurred between thromboplastin and heparin, the overall effect of the two on the clotting system agreeing with the values calculated from the mass law. Chargaff, Ziff & Cohen [1940] studied the same reaction isolated from the clotting system and found that in aqueous solution thromboplastin could be made by shaking to dissociate into cephalin and protein. They also found that when heparin was added to thromboplastin it displaced the cephalin in it. Our explanation would be that dissociation of cephalin and protein had occurred, followed by combination of heparin and protein. As the second compound would have a lower dissociation constant than the first, the reaction would proceed until the heparin had largely displaced the cephalin. Chargaff *et al.* [1940] found that this heparin-protein compound was inactive as an anticoagulant in thrombin-fibrinogen mixtures, although it possessed full activity in plasma. This indicates that the compound is itself inactive as an anticoagulant; its dissociation constant is, however, fairly high and hence it is completely dissociated in plasma. This appears to be a more reasonable interpretation of the data than the assumption that the heparin-protein compound formed by the reaction is itself an anticoagulant. Washing the precipitate at pH 5.0 suppressed dissociation of the heparin-protein in the wash water. The same results were obtained with a cephalin-histone complex, but in this case the heparin-histone compound was inactive in plasma. This was to be expected, since, as reported above, this compound has a low dissociation constant.

It is suggested that the view advanced as to the nature of the reaction of heparin and thromboplastin may be of value in considering all the reactions involved in the clotting system, i.e. that reversible dissociation of the various compounds involved may occur and that when several reactions are possible, that one will occur to the greatest extent which leads to the compound with the lowest dissociation constant. For example, Ferguson has postulated his 'cephalin availability' theory to explain the action of thromboplastin; according to this, thromboplastin serves to transfer cephalin to prothrombin. The idea of dissociable compounds presented here provides a mechanism for such a

theory. Further, trypsin and other proteolytic enzymes, by destroying the protein part of thromboplastin, would increase its dissociation and speed the transfer of cephalin to prothrombin, thus providing a mechanism for the action of trypsin on the compound, the practical significance of which Ferguson has stressed. In the case of those clotting factors which are enzymes, the theory becomes the well-known theory of Michaelis and Menten. From the foregoing it is evident (1) that this reaction of heparin should be considered in experiments requiring its use, and (2) that in studies on blood clotting, heparin activity may be affected by the presence of factors not usually considered.

SUMMARY

Heparin forms stable salts with many proteins. The resulting salt is markedly different in its properties

(isoelectric point, solubility, etc.) from the original protein. In solution, these salts show partial dissociation according to the mass law. The dissociation constant of the compound varies with the protein, and the dissociation is markedly affected by pH and the presence of other anions and cations. The formation of compounds with a sufficiently low dissociation constant is followed by the loss of biological activity on the part of the anticoagulant. The application of these results to quantitative studies on blood clotting is indicated.

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The Isolation of Behenic Acid from Refined Mona Wax

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Montan wax has been refined by treatment with chromic acid in glacial acetic acid solution [Marcusson & Lederer, 1931]. Mona wax isolated from Irish peat [Reilly & Wilson, 1940] was not changed by this mixture. A sulphuric acid-chromic acid mixture gave 72.5% of a pale yellow acidic wax. Its properties are compared with those of Mona wax in the following table:

	Mona wax	Refined Mona wax
Acid value	46	149
Saponification value	98	173
Ester value	52	24
Iodine value	30	5.5
M.P.	70°	73.5°

The acids were isolated by the usual procedure [Chibnall, 1934; Reilly & Wilson, 1940] and converted to their methyl esters. By a series of frac-

tional distillations *in vacuo*, the latter were separated into a number of fractions. From one of these, an acid was isolated whose equivalent weight and elementary analysis suggested that it might be behenic acid $C_{22}H_{44}O_2$.

Stohmann & Langbein [1890] found the m.p. of behenic acid, obtained by the reduction of ethyl erucate, to be 77–78°. The value obtained in the present investigation was 77.5°. A more recent determination on the synthetic acid by Francis, Piper & Malkin [1930] gave a value of 80.0°. To determine its identity, the acid was submitted for X-ray crystallographic examination. C. H. Carlisle and D. M. Crowfoot (see below) showed, by measurements of the B and C crystal spacings, that the acid was contaminated with 2.5–10% of $C_{20}H_{40}O_2$, and probably with traces of a higher acid, $C_{24}H_{48}O_2$.