THE PROTEIN MATTER OF BILE.*

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The protein matter is undoubtedly one of the more important constituents of the bile, but in spite of this fact, only a relatively small amount of work seems to have been done upon it. The small amount of the substance in the fluid, combined with the extreme difficulty of purification, has delayed exact investigation.

The early investigators, Fourcroy, Gmelin, Frommherz, Gugert, Simon, and Berzelius (1), each in turn, made a very superficial study of the properties of bile protein, without much attempt at its purification. Somewhat later, Landwehr (3) claimed that bile protein was a true mucin containing only non-reducing carbohydrates, because he found that boiling it with dilute acids failed to produce any substance which was capable of reducing alkaline copper sulfate. His "mucin" preparation upon analysis gave: C 53.09, H 7.6, O 24.41, N 13.8, and S 1.1 per cent. Later he changed his views and stated that the protein which he analyzed was a mixture of globulin and glycocholic acid. Paijkull (2) showed that if we accept the analysis of Landwehr, the latter's assumption of a mixture of globulin and glycocholic acid was practically an impossibility. Paijkull's own work gave him a product with C 51.67, H 6.88, N 16.09, and S 1.74 per cent. He concluded that bile protein is not a true mucin, although it has some of the properties of a mucin;

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he was inclined to the view that it is a nucleoalbumin, but admitted that he was unable to obtain agreement in his phosphorus determinations. Wahlgren (4), Galdi (5), and Cavazzani (6) have recently published individual views regarding the nature of bile protein with some agreement in their conclusions.

It will be seen that the nature of bile protein is still unsettled. Should it be classified as a glucoprotein, a nucleoprotein, or a phosphoprotein; or is it a mixture of two or more of the above? The purpose of this investigation may be stated as (1) the preparation of pure bile protein, followed by its analysis, and (2) the study of its decomposition products and properties in an attempt to classify it properly.

Methods and Results.

Fresh ox bile was filtered through cheese-cloth and used at once to provide against bacterial decomposition of the protein. Many methods of separation and purification were tried, but only the more successful ones will be detailed in this paper. Direct precipitation by adding dilute acid, followed by repeated redissolving and reprecipitation gave a product which still retained pigment. Separation of impurities from acid-precipitated protein by dialysis also proved unsuitable for obtaining a pure protein. Salting out by ammonium sulfate was tried with unsatisfactory results. The following methods gave a product free from bile acids and containing little impurity except traces of bile pigment.

Sodium chloride, with a small amount of HCl, was used to precipitate the protein. The salt and acid brought down a slimy green mass which settled to the bottom, leaving the serum part of the bile almost colorless. The serum was poured off. It was then found that the green mass readily dissolved when stirred with alcohol.

This unexpected result, the solution of the protein in alcohol, must be explained on the assumption that it dissolved under the influence of other constituents. The next step was the addition of acetone until precipitation took place. The small amount of HCl still present probably helped in this last precipitation. The protein was thus obtained as a somewhat green flocculent
precipitate; the greater part of the pigment and a large part of
the bile acids were left in solution. Some more of the pigment
was removed by extraction with acetone in a Soxhlet apparatus,
but after a short time the acetone extraction seemed to reach its
limit with color still present. The material was then removed
from the Soxhlet apparatus and washed with distilled water
until free from chlorides, then after washing repeatedly with
alcohol, and finally with ether, the protein was dried for several
days at about 40°. A trial Kjeldahl determination upon this
material gave only 10.8 per cent N. This pointed to a consider-
able admixture of bile acids which were removed as follows:
300 cc. of distilled water and 5 cc. of 10 per cent NaOH were
added to 5 gm. of the dry material. After agitation, complete
solution was effected. 600 cc. of 95 per cent alcohol were stirred
in and no precipitation took place. 1,500 cc. of acetone and a
few drops of dilute HCl brought down a flocculent precipitate
of protein. Most of the liquid was poured off and separation
was completed by the centrifuge. The protein precipitate was
well triturated with alcohol to remove traces of bile acid.
After separating once more with the centrifuge, the material
was washed with distilled water. It was washed several times
with water, then with alcohol and finally with ether, and dried
at about 40°. It will be referred to as protein No. 1 a.

Upon being dried the product became somewhat greenish in
color, but was free from bile acids. It was insoluble in water
and soluble in alkalies. It gave all the protein color reactions
more or less distinctly.

In the Kjeldahl determination.

0.25 gm.: 0.03387 gm. N = 13.55 per cent N.
0.25 " 0.03441 " " = 13.76 " " 

In the ash determination.

0.2 gm.: 0.0036 gm. = 1.8 per cent ash.

Attempts were made to remove the green pigment from some
protein No. 1a by direct extraction. All the well known or-
ganic solvents and some special solvents for bile pigment, pro-
vided they were such as could be used under the circumstances
were tried, with little success.

In order to obtain a product with less ash, a quantity of bile
protein, separated by the NaCl-HCl method, was freed from
bile acids and pigment as far as possible as in the preparation of protein No. 1a, but in addition to this, it was subjected to successive washings with 0.002 N HCl. This was followed by washings with 0.001 N HCl and then several washings with distilled water to free from chlorides. In each washing, separation was effected by the centrifuge. If the isoelectric point of the protein was slightly on the acid side of neutrality, as its solubilities would lead one to expect, such treatment should free the material from metallic ions in accordance with the theory of Loeb (7), and the experience of Van Slyke and Baker (8) and Field (9). The resulting product was greenish in color, but free from bile acids. It will be referred to as protein No. 1b.

An ash determination gave 1.5 per cent. Some calcium was removed by the acid dialysis. The ash contained some phosphates, and traces of iron were detected.

In the Kjeldahl determination,

\[
0.25 \text{ gm.}: 0.03367 \text{ gm. } N = 13.47 \text{ per cent } N.
\]

After preliminary trials, in which the ordinary methods for determining phosphorus were tried, it was found that a micro method was essential. The method of Wolf and Österberg (10), in a modified form, was finally adopted. This is a combination of Benedict's (11) well known sulfur method, and that of Neumann (12) for phosphorus. The micro method of Raper (13) was substituted for the method of Neumann in my work. The method thus modified was rather laborious, but had an advantage in its economy in material required, since the same sample served for both S and P determinations. In the determination of phosphorus, the product weighed is lead molybdate, from which the phosphorus is calculated by multiplying by the factor 0.00703.

1 gm. protein: 0.0874 gm. BaSO₄,
Control with reagents: 0.0030 " "

\[
0.0844 " = 0.0116 \text{ gm. } S = 1.16 \text{ per cent } S.
\]

1 gm. protein: 0.1080 " lead molybdate.
\[
0.1080 \times 0.00703 = 0.000759 \text{ gm. } P = 0.075 \text{ per cent } P.
\]

In later analyses of the same protein by the Raper method direct, omitting the sulfur determination,

0.25 gm. protein: 0.0001767 gm. P = 0.070 per cent P.
0.25 " " 0.0001879 " " = 0.075 " " "
The average for these three determinations gave 0.073 per cent P.

In the combustion for C and H.

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 gm. protein:</td>
<td>0.2805 gm. CO₂ and 0.0925 gm. H₂O.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>“</td>
<td>0.2824</td>
<td>“</td>
<td>“</td>
</tr>
<tr>
<td>0.15</td>
<td>“</td>
<td>0.2771</td>
<td>“</td>
<td>“</td>
</tr>
<tr>
<td>0.15</td>
<td>“</td>
<td>0.2765</td>
<td>“</td>
<td>“</td>
</tr>
<tr>
<td>Average.......</td>
<td>0.2791</td>
<td>“</td>
<td>“</td>
<td>“</td>
</tr>
</tbody>
</table>

= 50.75 per cent C and 7.02 per cent H.

The results of the analyses of protein No. 1 b may be summarized as follows: C 50.75, H 7.02, N 13.47, S 1.16, P 0.07, and ash 1.50 per cent.

After completion of analysis of protein No. 1 b, it was determined to try to obtain a product comparatively free from pigments as well as bile acids.

It had been noticed that some chloroform, which had been added as a preservative to a suspension of partially purified protein, had become highly colored. This suggested chloroform as an extractor for the pigment. It had already been tried on dried material without result. Upon experiment it was found that chloroform would dissolve out the pigment best in a slightly acid medium. Efforts were made to ascertain the exact acidity at which the color would leave the protein and pass to the chloroform upon shaking, but it was soon found that the acidity was not the only factor involved. When the protein was precipitated by acid alone, the chloroform failed to extract the pigment. On the other hand, when it was precipitated by acid alcohol, most of the pigment was more readily extracted.

A quantity of ox bile was mixed with an equal volume of 95 per cent alcohol which had been acidified with sulfuric acid. The protein so precipitated was immediately separated, to avoid denaturation by the alcohol. It was then dissolved in water and repeatedly shaken with fresh lots of chloroform and small amounts of acid. Most of the pigment passed into the chloroform. The resulting protein, which had gradually become less soluble in water, was dissolved in dilute sodium hydroxide, salted out by sodium sulfate, and dialyzed against distilled water. It was then washed with alcohol and after extraction with alcohol in
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the Soxhlet apparatus, washed with ether and dried. The resulting material was still somewhat stained with pigment. Upon analysis of this protein No. 2,

0.12 gm. protein: 0.2080 gm. CO₂ and 0.0762 gm. H₂O.
0.12 " " 0.2110 " " 0.0795 " "
0.12 " " 0.2105 " " 0.0787 " "

Average........0.2098 " " 0.0781 " "

= 47.68 per cent C and 7.23 per cent H.

In the ash determination.

0.12 gm. protein: 0.0025 gm. = 2 per cent ash.

Up to this time, extraction of pigment with chloroform after initial precipitation by acidified alcohol, had given the most promising results in removal of pigment. Further investigation along this line seemed warranted. The fact that protein that had been precipitated by alcohol would give up pigment to chloroform in acid solution, whereas protein precipitated by acid refused to do so, seemed to indicate that in some manner the alcohol had been assisting the chloroform in the extraction in the former experiment with protein No. 2.

95 per cent alcohol and chloroform mixed together were added to bile and the test-tube was shaken. After the separation into the chloroform-alcohol phase below, and the water-alcohol phase above, it was found that very little pigment had left the water-alcohol phase. However, when a little acid was added, and the shaking was repeated, upon separation of the layers, nearly all the pigment was in the chloroform-alcohol below. The water-alcohol phase above was only slightly greenish. The protein was floating as a precipitate at the top of the heavier chloroform layer. It was found that a mixture of 1 volume of chloroform with 3 volumes of 95 per cent alcohol gave good results. To 40 cc. of this mixture, 1 cc. of 25 per cent H₂SO₄ was added and this was mixed with an equal volume of bile. A precipitate of protein and some bile salts settled on the top surface of the dark colored chloroform layer. The whole was transferred to a separatory funnel and after a few moments, the chloroform, highly colored with green pigment, was drawn off. Since it carried some of the alcohol with it in solution, the next addition of chloroform contained some alcohol to maintain the proportion suitable for
further extraction of the pigment. It was also desirable to keep the bile acids in solution as much as possible, and the extra alcohol tended towards this end. The extraction of the precipitate was repeated in this manner as long as color continued to pass into the chloroform layer. The result was an almost pure white protein somewhat contaminated with bile acids, but giving protein tests well.

The whole experiment was repeated, using 500 cc. of bile with 375 cc. of alcohol, 125 cc. of chloroform, and 13 cc. of \( \text{H}_2\text{SO}_4 \). As before, the alcohol, chloroform, and acid were mixed first and then added to the bile. The temperature rise was not sufficient to promote greatly denaturation of the protein. After repeated treatment with more chloroform and alcohol till pigment was no longer extracted, and the protein product was almost pure white, the chloroform was removed and alcohol added. The material was washed several times with alcohol, then with ether. The ether washings continued to show a slight amount of color, so the material was extracted with ether for several days in a Soxhlet apparatus. It was then dried and found to be free from bile pigment. It was a grayish white powder, by far the best product obtained. However, upon an ash determination, it was found highly contaminated with calcium sulfate, the calcium of the bile having been carried right through the purification process in the form of its sulfate (from the sulfuric acid added). The continued use of alcohol had favored this, as it is well known that calcium sulfate is particularly insoluble in alcohol. (For this reason, in subsequent preparations by the alcohol-chloroform method, it was decided to use hydrochloric acid in place of sulfuric.) The calcium was removed by dialysis in collodion bags against distilled water in a continuous dialyzing apparatus for a considerable time. The protein was then removed from the collodion bags, centrifuged, and washed repeatedly with portions of alcohol. It was then washed with ether and dried at a low heat, about 30-35\(^\circ\)C, for several days. It will be referred to as protein No. 3 a. Upon analysis,

\[
\begin{align*}
0.12 \text{ gm.:} & \quad 0.2018 \text{ gm. CO}_2 \text{ and } 0.0733 \text{ gm. H}_2\text{O} \\
0.12 \text{ "} & \quad 0.2042 \text{ " } 0.0782 \text{ " } 0.0782 \text{ " } 0.0782 \\
\text{Average } & \quad 0.2030 \text{ " } 0.0758 \text{ " } 0.0758 \text{ " } 0.0758 \\
& = 46.1 \text{ per cent C and } 7.0 \text{ per cent H.}
\end{align*}
\]

There was too little material for the analysis for other elements.
Another protein preparation had been carried on simultaneously with a slight modification of the above method in that the proportion of the alcohol-chloroform mixture was somewhat increased.

14 cc. of chloroform were mixed with 36 cc. of 95 per cent alcohol and 1 cc. of 25 per cent sulfuric acid was added, giving about 50 cc. in all. With this, 25 cc. of bile were mixed. 8 liters of bile were treated, using these proportions. The remainder of the process was essentially the same as in the preparation of No. 3 a.

After prolonged extraction of the product with alcohol in the Soxhlet apparatus it was washed with ether and air-dried. It was then dried in vacuo over sulfuric acid for 10 days at about 35°C. The final product (No. 3 b) was a grayish white powder, slightly hygroscopic, but nearly insoluble in water; insoluble in dilute acids; but soluble in stronger acid. It only partially dissolved in strong acetic acid. It was partially soluble in dilute alkali, forming an opalescent solution. The solution in dilute alkali showed little or no evidence of the mucilaginous consistency which is so characteristic of the native mucins and of the bile itself. This protein, No. 3 b, represents, I believe, the purest product obtained; it was therefore the most valuable for elementary analysis.

In the ash determinations.

<table>
<thead>
<tr>
<th>0.1 gm. protein</th>
<th>0.0011 gm.</th>
<th>= 1.1 per cent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.0010</td>
<td>1.0</td>
</tr>
<tr>
<td>0.096</td>
<td>0.0012</td>
<td>1.2</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>1.1</td>
</tr>
</tbody>
</table>

In the combustions.

<table>
<thead>
<tr>
<th>0.1 gm. protein</th>
<th>0.1664 gm. CO₂ and 0.0693 gm. H₂O.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.1625                 0.0691</td>
</tr>
<tr>
<td>Average...........</td>
<td>0.1645                 0.0692</td>
</tr>
<tr>
<td>= 44.85 per cent C and 7.70 per cent H.</td>
<td></td>
</tr>
</tbody>
</table>

The figures seemed unexpectedly low for C and too high for H, so a moisture determination was carried out. 0.1 gm. was dried at 90–100°C and, after cooling in a sulfuric acid desiccator, it was weighed. Its dry weight was 0.096 gm., a loss of 4 mg. It gained weight rapidly on exposure to the air and soon reached
its former weight. The hygroscopic nature of the material had never, up to this time, been so evident.

Deducting the 4 mg. of moisture in the above combustions on protein No. 3b,

\[
\begin{align*}
0.096 \text{ gm. protein}: & \ 0.1664 \text{ gm. CO}_2 \text{ and } 0.0653 \text{ gm. H}_2\text{O}. \\
0.096 \ " & \ 0.1625 \ " \ " \ 0.0651 \ " \ " \\
\text{Average.........} & \ 0.1645 \ " \ " \ " \ 0.0652 \ " \ " \\
= 46.72 \text{ per cent C and 7.24 per cent H.}
\end{align*}
\]

In the Kjeldahl determination.

\[
\begin{align*}
0.12 \text{ gm. protein}: & \ 0.016548 \text{ gm. N}. \\
0.12 \ " & \ 0.016520 \ " \ " \\
\text{Average.........} & \ 0.016534 \ " \ " \ = 13.78 \text{ per cent N.}
\end{align*}
\]

In the Wolf and Österberg method for S and P.

\[
\begin{align*}
0.25 \text{ gm. protein}: & \ 0.0288 \text{ gm. BaSO}_4. \\
0.25 \ " & \ 0.0276 \ " \ " \\
\text{Average.........} & \ 0.0282 \ " \ " \ = 1.12 \text{ per cent S.}
\end{align*}
\]

\[
\begin{align*}
0.25 \text{ gm. protein}: & \ 0.0819 \text{ gm. PbMoO}_4 = 0.230 \text{ per cent P}. \\
0.25 \ " & \ 0.0780 \ " \ " \ = 0.219 \ " \ " \\
\text{In another P determination, by the method of Raper direct (omitting the S determination)}. \\
0.25 \text{ gm. protein}: & \ 0.0847 \text{ gm. PbMoO}_4 = 0.238 \text{ per cent P}. \\
\text{Average........................} & \ 0.23 \ " \ " \ " \\
\end{align*}
\]

Controls were unsatisfactory, but there was a trace of P in reagents.

The analysis of protein No. 3b may now be summarized as follows: C 46.72, H 7.24, N 13.78, S 1.12, P 0.23, and ash 1.10 per cent.

As will be shown later in this paper, it seems probable that the bile contains at least small amounts of a second protein. When one considers the prolonged treatment necessary for the isolation of the pure protein material, and the difference in the methods, it is quite likely that the final mixtures as analyzed may not contain the same proportion of the two original proteins as they occur in the bile. This would account for the difference in C content. The presence of traces of pigment in No. 1 b would also promote a slight tendency towards a higher C analysis.
The refractive index of alkali solutions of the protein was studied, using an Abbé refractometer and the monochromatic light of the sodium flame. According to an extensive investigation of the refractive index of various proteins by Robertson (14), the addition of relatively small amounts of a protein to a dilute acid, base, or other solvent, changes the refractive index of the solvent.

In the work with this denatured bile protein, 0.02 N NaOH was used as the solvent. The temperature was about 25°C. As already stated, the protein was only partially soluble in dilute alkali. Mixtures of different proportions of alkali and protein were made up, shaken, and allowed to stand for some time in order to dissolve all the soluble part. The insoluble portion settled out and the opalescent upper layer was used in taking the readings. No attempt was made to ascertain the exact amount of protein in solution.

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein-alkali mixture.</th>
<th>Mean refractive index.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control, 0.02 N NaOH.</td>
<td>1.3332</td>
</tr>
<tr>
<td>2</td>
<td>0.25 per cent protein.</td>
<td>1.3337</td>
</tr>
<tr>
<td>3</td>
<td>0.50 “ “ “</td>
<td>1.3341</td>
</tr>
<tr>
<td>4</td>
<td>0.75 “ “ “</td>
<td>1.3345</td>
</tr>
<tr>
<td>5</td>
<td>1.00 “ “ “</td>
<td>1.3349</td>
</tr>
</tbody>
</table>

The proteins so far prepared, Nos. 1 a, 1 b, 2, 3 a, and 3 b, were all purified by a more or less prolonged treatment with alcohol. Therefore, they were all denatured to some extent. It seemed desirable to prepare the bile protein in an unchanged form. While the denatured protein served for purposes of analysis, its insolubility in most solvents rendered it of little use for the study of its physical properties. According to the best authorities, denaturation is a form of dehydration. It is a distinct chemical change with a very high temperature coefficient. Chick and Martin (15) found that in the presence of an excess of water, the process of denaturing follows the laws of a monomolecular reaction provided the hydrogen ion concentration is kept constant. According to the investigations of Hofmeister, Bovie (16), and others, and more recently, Young (17), at least three things should be avoided in the preparation of an undenatured
protein; viz., (1) high temperature, (2) the chemical rays of sunlight, and (3) prolonged contact with dilute acids or bases, alcohol or acetone.

In the following method a low concentration of slightly acidified alcohol was used with chloroform as an extractor of bile pigment. The precipitated protein was separated from the alcohol as quickly as possible. Prolonged dialysis against cold distilled water was relied upon to complete the process of purification.

25 cc. of chloroform were mixed with 25 cc. of 95 per cent alcohol and 0.3 cc. of concentrated HCl was added. The whole was mixed with 50 cc. of bile in a separatory funnel. This caused a precipitation of at least most of the protein and gave a good separation of pigment. After immediate removal of the chloroform layer, the protein was again shaken with 10 cc. of fresh chloroform. This was repeated with another 10 cc. of the same solvent. Most, but not all the color was thus extracted, giving a fairly white precipitate of protein with some bile acids. Since the experiment looked promising, the remaining 950 cc. of 1 liter of ox bile were treated in a similar manner with corresponding proportions of alcohol, chloroform, and hydrochloric acid. The product was highly contaminated with bile acids. In previous work, diffusion of these acids through parchment had proved extremely slow. However, Paijkull (2) claims to have succeeded in practically eliminating bile acids in this way. In the writer's work, one portion was dialyzed in the continuous dialyzing apparatus for several weeks during laboratory hours. The flask containing the protein in collodion bags was surrounded with colored paper as a protection against the chemical effect of sunlight. The other portion of protein was dialyzed in collodion bags for about 2 months against distilled water in a dark closet. In both portions, a product was obtained nearly free from bile acids (according to the Pettenkofer test), but some pigment still persisted. No analysis of this protein (No. 4) was undertaken. It was, perhaps, somewhat denatured from the long treatment, but apparently much less so than the former preparation because it was more soluble in dilute NaOH and almost completely soluble in excess of acetic acid. However, it was practically insoluble in water and was precipitated from
alkaline solution upon neutralizing or making slightly acid. It gave positive xanthoproteic and Millon's tests, and responded slowly to the biuret reaction.

According to Hammarsten (18) mucins and nucleoproteins are insoluble in water, except in the form of their alkali salts; i.e., in the presence of traces of alkali. If this be the case, the insolubility in water of the last product is not surprising. In order to explain the solution of the native protein in the bile, we may assume that like the bile acids, it exists in solution in the form of the calcium or sodium salt. However, it is quite possible that it is not in true solution at all. The protein and the bile salts may have a mutual effect upon one another in maintaining their natural dissolved condition. Freezing point determinations, with concurrent analyses of the bile, might throw some light upon this subject, but very little work along this line has been done. Von Rentkowski (19) working with human bile, found that the freezing point was about $-0.543^\circ$. This rather slight depression of the freezing point is difficult to reconcile with the comparatively large proportion of salts (organic and inorganic) which are found in the bile. Hence one is inclined to believe that the rather large proportion of organic bile salts (sodium glycocholate and sodium taurocholate) is not producing its maximum effect upon the freezing point. This may best be explained by the assumption that a portion of the bile salts is not in true solution, but is adsorbed upon the protein.

Beside the hypothesis that the material is a nucleoprotein, there are two other alternatives. The phosphorus of the analyses may all be due to the inorganic or organic impurities, in which case the protein must be classed as a glucoprotein; or, the material may be a phosphoprotein (nucleoalbumin) with only a low percentage of phosphorus.

The following experiments were carried out to ascertain the nature of the protein of ox bile:

1. Some protein was kept in 1 per cent NaOH for 24 hours; no phosphorus test with ammonium molybdate was obtained. According to Plimmer and Scott (20), this indicates that it is not a phosphoprotein.

The solution of the problem seemed to hang upon a study of the products of hydrolysis. According to the work of Müller
(21) and his pupils, the true mucins contain a polysaccharide which is easily broken down by boiling with dilute acids. A reducing body is formed, which is glucosamine or a related amino sugar.

2. About 3 gm. of fairly well depigmented protein (which had been precipitated by alcohol) were heated for 20 hours on a boiling water bath with 30 cc. of 10 per cent HCl. A reflux condenser kept the volume constant. A large part of the protein failed to dissolve, but remained in the form of dark sticky lumps. The product was filtered and the filtrate showed no reducing action upon alkaline copper sulfate. Similar experiments with 2.5 per cent HCl, concentrated HCl, and 10 per cent HCl and dilute H$_2$SO$_4$ resulted in failure to produce a reducing sugar.

It is evident from the results of the above experiments that bile protein either does not contain a reducing sugar of the glucosamine type, or if such a body is present, it is very difficult to release by the usual procedure of acid hydrolysis. It should be noted here that Paijkull (2) had a similar experience in his attempts to obtain a reducing substance from bile protein; Landwehr (3), as stated earlier in this article, explained this lack of reducing power in the products of hydrolysis by assuming that the carbohydrate in bile protein was of such a character that it could not furnish any reducing substance.

Bile protein treated with dimethylaminobenzaldehyde after the manner of Ehrlich gave a rose color, a reaction said to be positive with all glucoproteins.

Some further hydrolysis experiments were then carried out. Levene and La Forge (22), in their work on the mucoids, brought about incipient hydrolysis by allowing the protein to stand for some hours in dilute alkali; e.g., 2 per cent KOH or lime-water. Some partially purified bile protein was placed in 2 per cent NaOH at about 40°C. for a short time and then left at room temperature for about a week. The product was then neutralized with HCl and excess acid was added to make about a 10 per cent solution of HCl. After boiling about 3 hours the solution was found to have slight reducing power by the usual test.

It would seem from this last experiment that the dilute alkali had some hydrolyzing effect that acid treatment alone could not produce. The fact that the reducing power was so small may
be interpreted in two ways: \((a)\) If the bile protein were a chemical individual and all its molecules capable of furnishing a reducing body, then only a few of them were attacked by the alkali. \((b)\) On the other hand, this slight reducing power might come from the hydrolysis of a second protein which represented only a small part of the total protein present.

During the hydrolysis experiments, tests were made for the presence of purine bases in the various solutions. The ammoniacal silver nitrate test appeared to be slightly positive after standing several hours, but the amount of the brown precipitate was too small for confirmation. The same precipitate was again formed upon repeating the test with some other material. Other purine tests gave negative results.

Pyrimidine bases were not present in the free state as judged by qualitative tests. However, since they are more difficult to split off from the nucleic acid than the purine bases this is not surprising.

The evidence points to the presence of a small admixture of nucleoprotein with the main body of protein as separated from the bile.

Some purified bile protein was placed in a 30 per cent nitric acid solution and kept at about 35°C. for several days. The molybdic acid-phenylhydrazine test for phosphates was then applied. No separation of phosphates as a result of the nitric acid treatment could be detected. According to Macallum (23), nucleoproteins split off phosphorus under such treatment.

**CONCLUSIONS.**

The positive Molisch and Ehrlich reactions and the N content of 13.5 to 13.8 per cent point to the glucoprotein molecule; nucleoproteins usually show from 15 to 17 per cent N. The very low P content (0.07 to 0.23 per cent) as shown by analysis and the negative result of the test for P after the action of 30 per cent HNO₃ seem to exclude the possibility of more than traces of nucleoprotein being present. The negative result of the Plimmer test with 1 per cent NaOH and the low P content certainly do not indicate that the material contains any large proportion of a phosphoprotein. The apparently faintly positive test for purines is evidence for the presence of traces of nucleo-
protein; the difference in C content of products which had been precipitated and purified by widely different procedures can best be interpreted by the assumption that a mixture of proteins is found in ox bile. The solubilities of the material furnish no clue whatever, and the lack of any considerable amount of reducing carbohydrate in the hydrolyzed material remains unexplained. Nevertheless, the bulk of the evidence seems to point to a mixture of a large proportion of some very stable form of glucoprotein with a much smaller amount of nucleoprotein.

SUMMARY.

1. The protein of ox bile has been isolated and purified according to several different methods; many other procedures along this line have been extensively investigated.
2. Three of the above methods gave a product which was undoubtedly denatured; the fourth we believe to be unchanged, or at least very slightly denatured.
3. Elemental analyses have been carried out upon two of these purified products for O, H, N, S, and P. C and H have also been determined with other products obtained by more or less modified procedures.
4. A study of the reactions and of the products of hydrolysis of ox bile protein has been made with a view to establishing the nature of the substance.
5. Evidence has been gathered which tends to show that the protein of ox bile is a mixture of a comparatively large proportion of glucoprotein with a small amount of nucleoprotein.

In conclusion, I desire to express my thanks to Prof. A. B. Macallum for many valuable suggestions in connection with the carrying on of this work.

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