Analysis of the Carbon Monoxide Product

It was recently reported that CO hemoglobin is relatively more resistant than oxyhemoglobin to digestion by the pancreatic enzymes (1), and that a heme-protein fragment can be precipitated either from the digest solution or from the dialyzed digest by acidification with acetic acid (2). The composition of this CO product has now been investigated.

Iron Content—The value of the iron to nitrogen ratio,\(^1\) which in early experiments lay near 0.43, was found to vary, figures of 0.39, 0.33, and even 0.21 occurring with later preparations. This ratio is independent of the method by which the product is isolated from the digest solution. Three samples from the same digest, (a) precipitated by acetic acid after dialysis of the digest for 1 day, (b) precipitated after dialysis for 6 days, and (c) precipitated directly without dialysis and washed several times with water, had values of 0.254, 0.258, and 0.263 respectively. The variation in different lots may be due to a gradual inactivation of the crude pancreatin mixture employed (cf. Haurowitz (3)).

A sample of CO product with an iron to nitrogen ratio of 0.432 was found to have an arginine content equivalent to one arginine residue per 10.3 atoms of iron. If the protein fragment of the CO product were a low molecular polypeptide, representing the original heme-protein linkage, with arginine as one of its constituent

\(^1\) This figure represents a ratio by weight.
residues, then a minimum ratio of one arginine residue per 4 atoms of iron would be required.

Amino Acid Distribution—Samples of CO product have been analyzed for their amino acid constituents, and the results obtained, along with a typical analysis of hemoglobin, are presented in Table I. Table II presents a comparison of the composit-

<table>
<thead>
<tr>
<th>Nitrogen Distribution in Humin-Free Hydrolysate from CO Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Fe:N value</td>
</tr>
<tr>
<td>Hydrolysate N taken, gm</td>
</tr>
<tr>
<td>Histidine N, %</td>
</tr>
<tr>
<td>Arginine &quot; %</td>
</tr>
<tr>
<td>Lysine N, %</td>
</tr>
<tr>
<td>Aspartic acid N, %</td>
</tr>
<tr>
<td>Glutamic acid &quot; %</td>
</tr>
<tr>
<td>Total N recovered, %</td>
</tr>
</tbody>
</table>

* Values for hemoglobin are taken from Bergmann and Niemann (4).
† Lost.

<table>
<thead>
<tr>
<th>Comparison of Nitrogen Distribution of CO Product and Hemin Proteose of Haurowitz (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Basic amino acid</td>
</tr>
<tr>
<td>Ammonia</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
</tbody>
</table>

The facts that the distribution of nitrogen among the amino acids accounted for is like that of hemoglobin except for the variation in arginine and lysine, and that the total recovered nitrogen is of the same order of magnitude for both the CO product and hemoglobin indicate that the protein fragment is not a low molecular polypeptide. It is evident that the belief expressed earlier re-
garding the character of the CO product is no longer tenable, for any stabilizing influence which carbon monoxide may have on the heme-globin linkage is apparently insufficient to prevent a breakdown of that linkage during pancreatic hydrolysis.

*Pancreatic Digestion of Hemoglobin, Methemoglobin, Oxyhemoglobin, and Carboxyhemoglobin*

The digestion of CO hemoglobin by a crude pancreatin extract has been found to proceed less rapidly than that of oxyhemoglobin (1). In order to determine the behavior of reduced hemoglobin and methemoglobin relative to the oxy and carboxy forms, the simultaneous digestion of all four proteins by pancreatin has been investigated.2

The data obtained from these studies are charted in Fig. 1, with a similar curve for globin. It is seen that CO hemoglobin is the most resistant to attack by the enzymes, and that reduced hemoglobin approaches it in this respect, being the least rapidly attacked of the remaining forms. At the beginning of the digestion methemoglobin is more quickly split than is oxyhemoglobin; later its rate is about the same as that of oxyhemoglobin. After 7 to 10 days all of the digests approach the same stage, 0.46 ± 0.03 for the amino to total nitrogen ratio.

---

2 One of the authors (W. F. R.) wishes to express his indebtedness to Miss Elisabeth L. Johnson for her assistance with the enzyme studies.
Factors other than the condition of the protein substrate do not appear to influence the rates of reaction. The differences between oxy- and CO hemoglobin appear at both acid and basic pH. CO does not retard the splitting of gelatin (1), nor does the presence of CO hemoglobin diminish the rate at which globin is digested by pancreatin; i.e., CO hemoglobin does not inhibit the enzymes. This is shown by the experiment reported in Table III, in which it was found that the increase in amino nitrogen in a mixed digestion of CO hemoglobin and globin was equivalent to the average of the increases in separate digestions of these two substrates. The use of a homogeneous crystalline enzyme, chymotrypsin, instead of the crude pancreatin mixture does not influence the results (Fig. 2).

TABLE III

Digestion of Globin by Pancreatin in Presence of CO Hemoglobin

<table>
<thead>
<tr>
<th>Time</th>
<th>COHb (1)</th>
<th>Globin (2)</th>
<th>1:1 mixture of COHb and globin</th>
<th>Arithmetical mean, (1) and (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td>Ratio, amino to total N</td>
<td>Ratio, amino to total N</td>
<td>Ratio, amino to total N</td>
<td>Ratio, amino to total N</td>
</tr>
<tr>
<td>0</td>
<td>0.064</td>
<td>0.070</td>
<td>0.064</td>
<td>0.070</td>
</tr>
<tr>
<td>6</td>
<td>0.11</td>
<td>0.18</td>
<td>0.143</td>
<td>0.19</td>
</tr>
<tr>
<td>19</td>
<td>0.13</td>
<td>0.27</td>
<td>0.153</td>
<td>0.19</td>
</tr>
<tr>
<td>24</td>
<td>0.14</td>
<td>0.36</td>
<td>0.173</td>
<td>0.22</td>
</tr>
<tr>
<td>71</td>
<td>0.23</td>
<td>0.42</td>
<td>0.273</td>
<td>0.28</td>
</tr>
</tbody>
</table>

The relative behavior of globin and hemoglobin might be expected from the work of Gralen (5), who found that globin has half the molecular weight of hemoglobin. It would therefore offer more surface for union with enzymes than would an equal weight of hemoglobin, thus being more rapidly digested.

If globin were a hemispherical body, two heme groups placed diametrically opposite on the semiplanar surface would enable union between two such halves to give a hemoglobin molecule in which four hemes are located in the central plane of a square. This arrangement would agree with the conclusion reached by Pauling (6) from the oxygen equilibrium data of hemoglobin. The two globin halves might then be held together both by the two coordinate valencies of the heme iron not saturated by por-
phyrin nitrogen (Conant (7)) and by a second kind of linkage, possibly hydrogen bonds, still active when the first type is occupied with a foreign molecule such as oxygen. Reduced hemoglobin having both kinds in force would thus be more resistant to breakdown than oxyhemoglobin. The stability of CO hemoglobin, however, could not be explained in this way.

The dissociation into half molecules undergone by CO hemoglobin in urea (8, 9) would depend upon the rupture of the subsidiary linkages. It would be valuable to determine whether this dissociation is dependent upon the state of the heme in a way corresponding to the digestion rates reported in this paper.

![Graph](http://www.jbc.org/)

**Fig. 2.** The digestion of oxyhemoglobin (○) and CO hemoglobin (△) by crystalline chymotrypsin at pH 7.5.

**EXPERIMENTAL**

**Analysis**

Preparation of the CO product was carried out as previously described (2). However, since it was found that direct precipitation from the digest solution resulted in the same product as that obtained after prolonged dialysis, the rather tedious work of dialyzing large quantities of solution was abandoned, and all products were prepared by precipitation with acetic acid, followed by thorough washing with water containing a few drops of acetic acid. The dry product was used for amino acid analyses. Iron determinations were made by the colorimetric method of Leavell and Ellis (10), total nitrogen analyses by the Kjeldahl method, and amino nitrogen analyses by the volumetric Van Slyke procedure. Amide
nitrogen was determined by hydrolysis of an aliquot of the dry product with 5 per cent HCl for 3 hours, followed by distillation of the liberated ammonia from the hydrolysate at pH 9 into standard acid.

The dicarboxylic acids were first precipitated from the humin-free hydrolysate as their calcium salts by the addition of alcohol and separated by the modified Foreman procedure developed in the laboratory of Chibnall (11). The original mother liquor from the alcohol precipitation and that from the reprecipitation of the calcium salts were combined and analyzed for the basic amino acids by the procedure of Tristram (12).

A sample of dry CO product weighing 5.17 gm. and containing 638 mg. of nitrogen, 163 mg. of iron, 20 mg. of amide nitrogen, and 27 mg. of amino nitrogen was hydrolyzed with 20 per cent HCl for 24 hours. The excess HCl was removed by repeated evaporation in a vacuum, and the humin filtered off and thoroughly washed with hot water. The combined filtrates, representing the total hydrolysate, contained 366 mg. of nitrogen.

The ammonia was removed from this hydrolysate by concentration in a vacuum at pH 8.9 and the dicarboxylic acids precipitated as their calcium salts by the addition of ethyl alcohol. These, after reprecipitation, contained 37 mg. of nitrogen, and 304 mg. of nitrogen remained in the filtrates.

l-Glutamic Acid—Glutamic acid hydrochloride was precipitated from the concentrated dicarboxylic acid fraction by saturation with HCl at -10°. The total yield of dry product was 0.085 gm.

C₆H₁₀O₄NCl. Calculated, N 7.6; found, N 7.8

l-Aspartic Acid—the filtrate from the glutamic acid hydrochloride, after removal of HCl, was treated with excess copper carbonate at 100°. The copper aspartate thus obtained weighed 0.314 gm. after equilibration with air.

C₄H₆NCu·4.5 H₂O. Calculated. N 5.07, Cu 23.0
Found. " 5.04, " 22.4

l-Histidine—After the removal of both calcium and chloride ions, histidine was precipitated as its silver salt at pH 7.3. The

* Nitrogen analyses on isolated amino acid salts were performed by the Dumas method.
precipitate was decomposed at pH 3.8 by the addition of H$_2$S, giving a solution containing 83 mg. of nitrogen. 0.927 gm. of histidine diflavianate was obtained from this solution, after concentration to 5 ml., by the addition of 1.25 gm. of flavianic acid.

(C$_{14}H_{29}N_3SO_4)$_2·(C$_4H_4N_3O_2$). Calculated, N 12.5; found, N 12.4

l-Arginine—From the concentrated mother liquor of the silver salt of histidine, arginine silver salt was precipitated at pH 12.5. This on decomposition with H$_2$S gave a solution containing 66 mg. of nitrogen, to which, after concentration to 8 ml., 0.360 gm. of flavianic acid in 2 ml. of hot water was added. The arginine flavianate after recrystallization weighed 0.458 gm.

(C$_{10}H_{14}N_3O_7$). (C$_6$H$_{14}$O$_2$N$_4$). Calculated, N 17.2; found, N 17.0

l-Lysine—Excess silver was removed by precipitation as the sulfide in acid medium, the solution concentrated to 30 ml., and lysine phosphotungstate obtained by the addition of 50 ml. of 20 per cent phosphotungstic acid in 5 per cent sulfuric acid. This was decomposed in the usual way, giving a solution containing 27 mg. of nitrogen. This solution was concentrated in the presence of BaCO$_3$ to 1 ml., diluted with 6 ml. of alcohol, and treated with 0.230 gm. of picric acid in alcohol at 0°. 0.151 gm. of lysine picrate was obtained.

(C$_4H_3O_7N_3$). (C$_6$H$_{14}$O$_2$N$_2$). Calculated, N 18.7; found, N 18.5

In all cases the mother liquors were worked up for additional product.

Digestions—The experimental procedure has already been described (1). For the digestions of Fig. 1 conditions were chosen so that the digest solution contained in 100 ml. 0.5 gm. of substrate nitrogen, 6.0 ml. of pancreatin solution similar to that used earlier, and 20 ml. of borate buffer, pH 8.0, saturated at room temperature. Both substrate and enzyme solutions were brought to 37° before being mixed.

Methemoglobin—A solution of oxyhemoglobin containing 27 mg. of nitrogen per ml. was oxidized at pH 8.5 to 9.0, with 1.1 equivalents of potassium ferricyanide. The resulting solution was then dialyzed against running water for 18 hours.

Hemoglobin—Oxyhemoglobin in a flask equipped with stopcocks was freed of oxygen by successive evacuations and flushings
with oxygen-free nitrogen. The digestion was carried out in the same apparatus, care being taken that no oxygen was introduced. The original purple color of reduced hemoglobin was still apparent after a week of digestion.

In the experiments of Fig. 2 chymotrypsin, prepared by the activation of twice recrystallized chymotrypsinogen ((13) p. 136), was employed. The activity of this as well as of the pancreatin solution used above had been determined by the procedure described by Northrop ((13) p. 155). In the digestions of Fig. 2 50 per cent more units of enzyme activity were employed than in those of Fig. 1.

SUMMARY

1. The product from the pancreatic digestion of CO hemoglobin has a variable Fe:N ratio, indicating that it is not a chemical unit.
2. The amino acid distribution in the protein part of this product does not support a low molecular polypeptide structure for it.
3. The following order has been found for the rates of digestion of several proteins related to hemoglobin: globin > methemoglobin > oxyhemoglobin > reduced hemoglobin > CO hemoglobin.

BIBLIOGRAPHY

7. Conant, J. B., Harvey Lectures, 28, 159 (1932-33).
THE HEME-GLOBIN LINKAGE OF HEMOGLOBIN: III. ANALYSIS OF THE CARBON MONOXIDE PRODUCT. THE PANCREATIC DIGESTIONS OF SEVERAL FORMS OF HEMOGLOBIN
William F. Ross and Richard B. Turner

J. Biol. Chem. 1941, 139:603-610.

Access the most updated version of this article at http://www.jbc.org/content/139/2/603.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/139/2/603.citation.full.html#ref-list-1