obtained. In common with other workers we find that methyl violet is the least inhibitory of the three dyestuffs.

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Received January 27th, 1956

The action of carboxypeptidase on different human haemoglobins

Most of the investigations of the eleven different human haemoglobins (A, B, C, D, E, F, G, H, I, J and K) described at the present have been carried out on normal adult (Hb-A), sickle-cell (Hb-B), Hb-C Hb-E and foetal haemoglobin (Hb-F). The results of these studies and especially of those concerning the N-terminal group analyses[1-4] have revealed that the molecules of these haemoglobins are built up by more than one polypeptide chain. According to the end-group analyses using Sanger's DNP method, Hb-A, Hb-B, Hb-C and Hb-E have 5 N-terminal valine residues and Hb-F two N-terminal valine residues. In the present paper some investigations are reported concerning the influence of carboxypeptidase on four different human haemoglobins (A, B, C and F) and what it reveals of the number, nature and distributions of C-terminal groups in these proteins.

The Hb-A was obtained from normal adults (laboratory workers); the Hb-B from patients with sickle-cell anemia and the Hb-C from patients with the homozygous Hb-C disease. These patients are described elsewhere[5,6]. Haemoglobin solutions were prepared and purified in the way described earlier[7]. The different haemoglobins were homogeneous in electrophoretic and chromatographic experiments[8]. The foetal haemoglobin was derived from samples of cord blood. A haemoglobin sample containing 96% Hb-F estimated by the alkali denaturation method[9] was used. Two samples of purified foetal haemoglobin, prepared by the alkali denaturation method of CHERNOFF[10], were also investigated. The time of denaturation was one minute (sample 1) and two minutes (sample 2). In both cases only foetal haemoglobin was present after this purification procedure.

The enzyme used during the course of this work was a preparation six times recrystallized obtained from Armour and Company. For qualitative studies 2.5 mg haemoglobin was incubated at 37° C for different periods (0; 6; 18; 27; 36; 72 hours) with 0.1 mg carboxypeptidase (enzyme-substrate mole ratio, 2:25) in 0.05 *M* phosphate buffer at pH 8.0 and 0.5% lithium chloride (final concentrations). An excess of di*iso*propyl fluorphosphate ($1.2 \cdot 10^{-5}$ m*M* of pure DFP per ml of solution) was added to destroy the last traces of endopeptidase activity in the carboxypeptidase preparation[11]. The final volume was 0.8 ml. The addition of 50 mg of Dowex-50 (H form, 20–50 mesh, 8% cross-linked) served to terminate the enzyme reaction by lowering the pH of the reaction solution to 2 to 3. The reaction products which were absorbed on the Dowex-50 resin were eluted with 5 *M* ammonia (0.2 ml) according to the molecular sieve method of PARTRIDGE AND THOMPSON[12,13] and subjected to paper chromatography. One-dimensional ascending paper chromatography (Whatman No. 1) was used with butanol-acetic acid-water (40:6:15) as solvent.

For quantitative analyses a hundred-fold enzyme substrate incubation mixture was prepared. The only difference was a lowered amount of the carboxypeptidase preparation (5 mg carboxypeptidase in 250 mg haemoglobin; enzyme-substrate mole ratio 1:25) and a corresponding lowered amount of DFP ($0.6 \cdot 10^{-5}$ m*M* per ml of solution). In general the same procedure was followed as described for the qualitative analyses. The mixture of amino acids finally obtained was freed from ammonia *in vacuo* and subjected to the quantitative column chromatographic method of STEIN AND MOORE[14] in the way described previously[7].

Using one-dimensional paper chromatography, two intensive spots and some faint but definite ones were obtained when haemoglobin A was incubated for six hours with carboxypeptidase. The two intensive spots corresponded to tyrosine and histidine, while the other ones were identical with leucine, alanine, glycine or glutamic acid. After longer periods of incubation (18, 36, and 72 hours) these and some other amino acids (phenylalanine, valine and lysine) were released in increased amounts. Approximately the same pictures were obtained when the haemoglobin from cord blood, Hb-B and Hb-C, was studied, while in similar experiments carried out with foetal haemoglobin, purified by the alkali denaturation method, the same amino acids were

released by the action of carboxypeptidase, the amount of most of them however being much increased.

The results of the quantitative estimations are recorded in Table I. The C-terminal amino acids, tyrosine and histidine, are completely released under the conditions of the experiment and attain an identical plateau value corresponding to one mole from one mole of haemoglobin. After prolonged digestion (36–72 hours), however, additional amounts of tyrosine and of histidine are released, especially from the purified foetal haemoglobin that was treated for two minutes with alkali. Many other amino acids are released successively at much slower rates than the two amino acids, tyrosine and histidine. These amino acids are also given in the table. As could be expected from the results obtained with the one-dimensional paper chromatogram, the amounts are much higher for the foetal haemoglobin that was treated by alkali. Increased values for many amino acids are also obtained from the samples of Hb-B and Hb-C.

From these results it may be concluded, that one mole tyrosine and one mole histidine are in C-terminal position in the four different human haemoglobins. Further interpretation of the reported data is rather difficult as the carboxypeptidase method is limited by different factors especially for the identification of C-terminal groups in proteins containing more than one polypeptide chain[15]. As the number of polypeptide chains in the haemoglobins is not known it may be possible that one or more C-terminal amino acids in these proteins may not conform to the specificity requirements necessary for reacting with the carboxypeptidase. This possibility is strengthened by the fact that the values of 2 C-terminal residues in normal adult haemoglobin and in some abnormal haemoglobins (B and C) do not agree with the number of 5 N-terminal valine residues found in these proteins[1–4]. In the case of foetal haemoglobin, however, the two C-terminal residues are connected with the two N valyl residues present in this protein[4], suggesting that two distinct polypeptide chains must exist in this haemoglobin. The data of the amino acids other than tyrosine and histidine released during the incubation of the haemoglobins with carboxypeptidase are very complicated and do not allow any conclusion to be drawn about the C-terminal sequence in the peptide chains of these proteins.

It may be obvious that the higher rates of release of the amino acids from purified foetal haemoglobin as compared with the haemoglobin from cord blood (with 96 % of Hb-F) is caused

TABLE I

THE RELEASE OF DIFFERENT AMINO ACIDS FROM THE HUMAN HAEMOGLOBINS STUDIED AFTER INCUBATION WITH CARBOXYPEPTIDASE

(The values are given in mole amino acid/mole haemoglobin, mol. weight 68,000)

	Time of incubation (hours)	*Tyrosine*	*Histidine*	*Leucine*	*Alanine*	*Valine*	*Phenylalanine*	*Glutamic acid*	*Glycine*	*Lysine*	*Methion.*	*Isoleucine*
Hb-A	6	0.66	0.57	0.04	0.04	0	0.04	0	0	0	—	—
	18	1.06	0.90	0.09	0.08	0.03	0.05	0.06	0.03	0.03	—	—
	36	1.04	0.86	0.13	0.15	0.17	0.07	0.07	0.05	0.05	—	—
	72	1.20	1.19	0.36	0.34	0.33	0.17	0.18	0.09	0.14	—	—
Hb-B	6	0.53	0.57	0.11	0.21	0.09	0.12	0.08	0.06	0.06	0	0
	18	0.90	0.81	0.39	0.29	0.29	0.21	0.10	0.25	0.19	0.12	0.04
	36	1.01	0.99	0.79	0.51	0.58	0.51	0.24	0.25	0.58	0.08	0.04
	72	1.02	1.09	1.63	1.51	1.19	0.77	0.56	0.55	1.26	0.18	0.08
Hb-C	6	0.68	0.64	0.23	0.08	0.22	0.11	0.16	0.09	0.09	0	0
	18	1.01	0.89	0.36	0.23	0.33	0.23	0.23	0.12	—	0.07	0.02
	36	1.05	1.01	0.94	0.72	0.57	0.93	0.39	0.23	0.35	0.10	0.05
	72	1.55	0.97	2.24	2.34	1.75	1.15	0.75	0.75	0.93	0.36	0.09
Hb from cord blood (96 % Hb-F)	18	0.93	0.85	0.21	0.06	0.09	0.17	0.08	0.03	0.03	0	0
	36	1.01	1.13	0.24	0.07	0.14	0.28	0.12	0.05	0.09	0.03	0.06
	72	1.06	1.00	0.36	0.18	0.21	0.34	0.15	0.11	0.16	0.06	0.08
Hb-F (one minute denaturation)	6	0.52	0.46	0.09	0.03	0.05	0.13	0.04	0.05	0.12	0.02	0.02
	18	1.04	1.04	0.94	0.44	0.59	0.47	0.08	0.07	0.49	0.26	0.07
	36	0.86	0.70	1.16	0.57	0.71	0.70	0.17	0.14	0.65	0.22	0.11
	72	1.68	1.54	3.07	2.47	2.13	—	0.50	0.59	2.55	—	0.33

by the short influence of alkali, which was used for the purification of this protein. Also the higher rates of release of amino acids from the Hb-B and Hb-C may be explained by a denaturation more or less of these proteins caused by their being kept longer before the experiment was carried out, since they were sent to us from the Netherlands Antilles (Curaçao).

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Received January 25th, 1956

The chemical structure of the reactive group of esterases

It is well known that a number of esterases are able to combine with $DF^{32}P^*$ to yield $D^{32}P$-proteins. At a recent symposium of the Faraday Society[1] it was reported that on mild enzymic hydrolysis by means of a polyvalent proteolytic pancreas preparation (cotazym) essentially one single radioactive $D^{32}P$-peptide can be obtained from each of these $D^{32}P$-proteins. It was assumed that these peptides would contain all or part of the original enzyme active site bound to the DP-radical and that therefore their analysis would provide valuable information on the chemical structure of this biologically very important site. Preliminary results were reported on DP-peptides prepared from the following esteratic proteins: true red cell cholinesterase, red cell ali-esterase, serum pseudo-cholinesterase, liver ali-esterase, α-chymotrypsin and trypsin. All peptides behaved in a very similar way during various electrophoretic and chromatographic procedures. The peptides obtained from DP-α-chymotrypsin, DP-trypsin and DP-liver-ali-esterase were examined in more detail; on acid hydrolysis they produced the amino acids reported in Table I (nos. 1, 2 and 3) in addition to DP. The sequence of the amino acids in the DP-peptide from chymotrypsin, reported earlier[1,2], was as follows: Gly. Asp. Ser. Gly. [Pro, Leu, (Gly)]. It will be noted that the composition of the trypsin peptide is in good agreement with the larger peptide recently isolated by DIXON *et al.*[3] by α-chymotrypsin hydrolysis of DP-trypsin.

In the present paper experiments are described which confirm and extend the preliminary results reported above. This was made possible by the development of new isolation techniques which will be described.

First optimal conditions for the cotazym hydrolysis at 37° C were established by running samples, taken at intervals from the incubation mixture, on a paper chromatogram using b.a.w. (butanol–acetic acid–water 4:1:5) as a solvent. Thus the time and pH necessary to split off the $D^{32}P$-peptides in optimal yield from the corresponding $D^{32}P$-proteins was found. The bulk of the incubation mixture was then hydrolysed accordingly. In the case of the $D^{32}P$-chymotrypsin the peptide was then isolated in the pure state as follows. The incubation mixture was first deproteinised by precipitation with alcohol 70% (v/v). The supernatant was concentrated *in vacuo* and chromatographed on a Dowex-50 column (8% cross-linkages 200–400 mesh) at pH 5.0 (Na-acetate buffer 0.1 *M*). The radioactive substance passed rapidly together with the acid amino

The following abbreviations will be used: DFP = di*iso*propylphosphorofluoridate; DP-, DP = di*iso*propylphosphoryl- and di*iso*propylphosphate respectively; DNFB = dinitrofluorobenzene; DNP- = dinitrophenyl-.