Evett, J., and Isenberg, I. (1969), *Ann. N. Y. Acad. Sci. 158*, 210.
Hayashi, H., and Iwai, K. (1971), *J. Biochem. 70*, 543.
Iwai, K., Ishikawa, K., and Hayashi, H. (1970), *Nature (London) 226*, 1056.
Job, P. (1928), *Ann. Chim. Paris 9*, 113.
Johns, E. W. (1971), *in* Histones and Nucleohistones, Phillips, D. M. P., Ed., London and New York, Plenum Press.
Johns, E. W. (1972), *Nature (London), New Biol. 237*, 87.
Laurence, D. J. R. (1966), *Biochem. J. 99*, 419.
Li, H. J., and Isenberg, I. (1973), *Biochim. Biophys. Acta* (in press).
Li, H. J., Wickett, R. R., and Isenberg, I. (1972), *Biopolymers 11*, 375.
Mauritzen, C. M., Starbuck, W. C., Saroja, I., Taylor, C. W., and Busch, H. (1967), *J. Biol. Chem. 242*, 2240.
Murray, K. (1964), *in* The Nucleohistones, Bonner, J., and Ts'o, P., Ed., San Francisco, Calif., Holden-Day, p 15.
Ogawa, Y., Quagliarotti, G., Jordan, J., Taylor, C. W., Starbuck, W. C., and Busch, H. (1969), *J. Biol. Chem. 244*, 4387.
Panyim, S., and Chalkley, R. (1969), *Biochemistry 8*, 3972.
Richards, B. M., and Pardon, J. F. (1970), *Exp. Cell. Res. 62*, 184.
Rossotti, F. J. C., and Rossotti, H. (1961), *in* The Determination of Stability Constants and Other Equilibrium Constants in Solution, New York, N. Y., McGraw-Hill.
Schaeppi, Y., and Treadwell, W. D. (1948), *Helv. Chim. Acta 31*, 577.
Senshu, T., and Iwai, K. (1970), *J. Biochem. 67*, 473.
Shih, T. Y., and Bonner, J. (1970), *J. Mol. Biol. 48*, 469.
Shih, T. Y., and Fasman, G. D. (1971), *Biochemistry 10*, 1675.
Simpson, R. T. (1972), *Biochemistry 11*, 2003.
Stellwagen, R. H., and Cole, R. D. (1969), *Annu. Rev. Biochem. 38*, 951.
Vosburgh, W. C., and Cooper, G. R. (1941), *J. Amer. Chem. Soc. 63*, 437.
Weber, G. (1952), *Biochem. J. 51*, 145.
Wickett, R. R., and Isenberg, I. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2687.
Wickett, R. R., Li, H. J., and Isenberg, I. (1972), *Biochemistry 11*, 2952.

Carboxymethylation of the Histidyl Residues of Insulin†

Italo Covelli,* Luigi Frati, and Jan Wolff

ABSTRACT: The reaction of [^{14}C]iodoacetate with insulin at pH 5.6 and 30° yields a carboxymethylated derivative in 13.8% yield provided zinc is first removed from the insulin. Zinc-insulin reacts very slowly. A pure product can be obtained in which the sole modification, as shown by amino acid analysis, is the carboxymethylation of the two histidyl residues of the B chain. This modification fails to alter the immunologic reactivity of insulin in standard radioimmunoassays but markedly reduces the biological activity of the derivative as measured by glucose oxidation or glycogen synthesis in the isolated rat diaphragm.

During studies on iodohistidine formation in simple proteins it became apparent that many histidine residues were difficult to iodinate, but that special cases existed in which electrostatic facilitation appeared to yield iodination rates that were comparable to those of exposed tyrosyl residues (Covelli and Wolff, 1966a,b; Wolff and Covelli, 1969). In insulin, iodination of one histidyl residue appeared to be controlled by the presence of zinc in the molecule (Covelli and Wolff, 1967). Since alkylation reactions proceed by similar electrophilic displacement and are also subject to factors such as electrostatic facilitation (Heinrikson *et al.*, 1965), we have investigated the reactivity of the two histidyl residues of the B chain (B^5 and B^{10}) toward iodoacetate at pH 5.6.

In the present work we describe the preparation and the purification of an insulin derivative, in which the sole modification was the N-carboxymethylation of the two histidyl residues.

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Materials and Methods

Crystalline bovine zinc insulin (25.4 IU/mg, Mann) with a zinc content of 0.48% and a moisture content of 5.2% was used. Zinc-free insulin was prepared according to a modification of the method of Sluyterman (1955; Covelli and Wolff, 1967). It contained less than 0.04% zinc, as calculated by the Versene titration method of Flaschka (1952), and a moisture content of 7.9%. Iodoacetic acid (Eastman) was recrystallized twice from petroleum ether (bp 30–60°) and the colorless crystals were dried *in vacuo* and stored at −20°. Iodoacetic acid-*1*-^{14}*C* (The Radiochemical Centre, Amersham, England) had a specific activity of 1.3 mCi/mmole. Glucose-*1*-^{14}*C* (The Radiochemical Centre, Amersham, England) and the Insulin Radioimmunoassay Kit (Wellcome, England, or Società Ricerche Nucleari, Italy) were also used. Cyanogum-41 was purchased from British Drug Houses. Ultra Pure urea was a product of Mann; it was stored at +4° and solutions were made immediately before use to minimize the formation of cyanate (Stark *et al.*, 1960). All other reagents were of analytical grade and triple-distilled water was used throughout.

Time Course of Alkylation. In a standard experiment the reaction mixture contained 6–8 μmol of insulin dissolved in 8

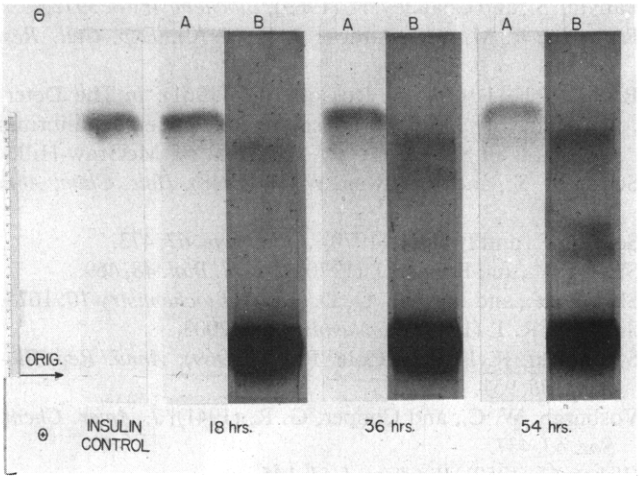

FIGURE 1: Time course of histidine carboxymethylation in zinc-insulin. Paper electrophoresis in 33% acetic acid as a function of time. (A) Electropherograms, protein stained with Naphthalene Black 10B; (B) autoradiograms; run 16 hr, 12 mA/strip.

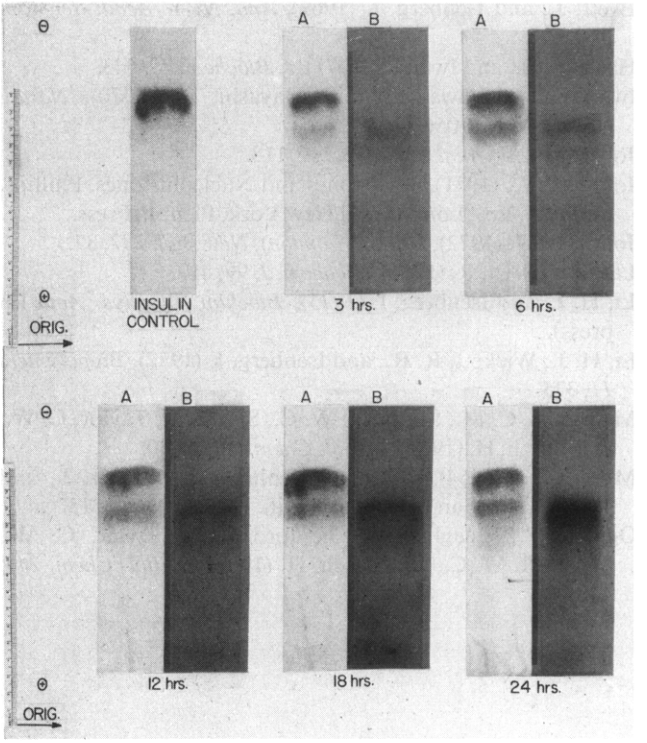

FIGURE 2: Time course of histidine carboxymethylation in zinc-free insulin. Conditions as in Figure 1.

ml of 0.2 M acetate buffer containing 3.2 M urea at pH 5.6. This concentration of urea was strong enough to dissolve the protein at this pH without causing any irreversible changes, as judged by its effect on glucose-*1*-^{14}C oxidation. ^{14}C-Labeled iodoacetic acid, dissolved in 2.0 ml of 0.2 M sodium acetate adjusted to pH 5.6, was slowly added to the protein solution to a molar excess of 20:1 and the mixture was allowed to incubate at $+30 \pm 1.5°$ in a covered Dubnoff metabolic bath and gently shaken (40 cpm). Preliminary experiments were carried out to optimize the molar ratio and the temperature of incubation. At regular intervals 0.3-ml samples were withdrawn and analyzed by paper electrophoresis in 33% acetic acid, as described by Sluyterman (1955).

Analytical Procedures. Analytical disc gel electrophoresis has been performed in 6% Cyanogum-41 using 0.1 M sodium citrate buffer (pH 3.8) in 4 M urea, for 90 min at 6 mA/tube. The gels were stained with Naphthalene Black 10B in 7% acetic acid and destained with 7% acetic acid.

Amino acid analyses of the insulin B chain were performed on a Carlo Erba automatic amino acid analyzer, according to Spackman *et al.* (1958). All hydrolyses were carried out in constant-boiling 6 N HCl in sealed, evacuated tubes for 36 hr. The isolated B chains were obtained either by sulfitolysis according to Dixon and Wardlaw (1960) or by reduction and alkylation of the cystine residues according to Anfinsen and Haber (1961) followed by column chromatography on Dowex 1-X2. The total recovery of B chains was about 35% in all cases.

1-*N*- and 3-*N*-carboxymethylhistidine have been synthetized as described by Crestfield *et al.* (1963), and the two isomers were separated by chromatography and used as reference compounds.

Biological and Immunological Assays. The biological activity of the purified CM-insulin[1] has been assayed by both stimulation of glucose-*1*-^{14}C oxidation (Field *et al.*, 1960), and ^{14}C-labeled glycogen synthesis in the rat diaphragm (Pastan *et al.*, 1966). In both cases insulin which had been subjected to the same manipulations, except for the alkylation, was used as control. The immunological reactivity of the purified CM-insulin was assayed according to Morgan *et al.* (1964).

Results

Time Course of Alkylation. The time course of the histidine alkylation in zinc-insulin is shown in Figure 1. In the electropherogram a slower cathodal migrating protein band is evident. This protein is radioactive, as demonstrated by autoradiography, and has subsequently been identified as CM-insulin. However, even after 54 hr only a small fraction of the total protein has reacted, and considerable quantities of by-products or other derivatives are present in the mixture. The radioactive spot closest to the origin is unreacted [^{14}C]-iodoacetic acid.

In order to increase the yield of the alkylation reaction we have used zinc-free insulin instead of the zinc-insulin. The time course of histidine alkylation in the zinc-free insulin is shown in Figure 2. It is evident that the efficiency of the reaction is significantly increased when zinc is removed. Twenty-four hours after the start of the reaction about 50% of the total protein has reacted and the fraction of radioactivity present as by-products or other derivatives is trivial.

Purification. Lyophilized zinc-free insulin (420 mg; 70 μmol) was dissolved in 30 ml of the buffer and treated for 18 hr with a 20-fold molar excess of labeled iodoacetic acid. At the end of incubation the protein was precipitated with ether and the precipitate was washed two times with 0.05 M acetate buffer at pH 5.0. The protein was dissolved in 5 ml of 0.1 M sodium citrate buffer (pH 3.8) containing 4 M urea and then subjected to a preparative polyacrylamide gel electrophoresis in a Buchler apparatus using 8% Cyanogum-41 as gel in a jacketed column maintained at $+4°$, at 60 mA for 6 hr. The buffer was 0.1 M sodium citrate with 4 M urea and the pH at the start was 3.8. Fractions of 5 ml were collected. The protein peaks were determined by the absorbance at 280 nm and the radioactivity was measured in a liquid scintillation counter. The

[1] Abbreviation used is: CM-insulin, *N*-carboxymethylhistidylinsulin.

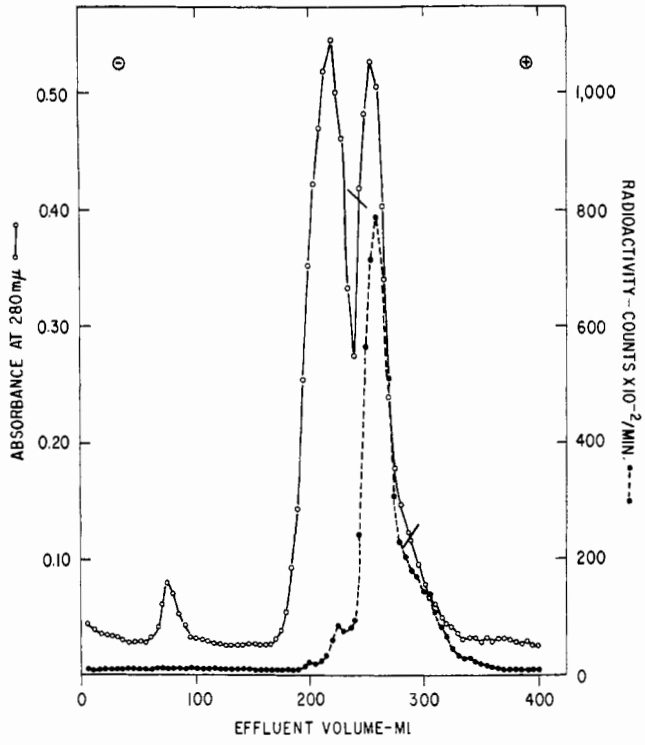

FIGURE 3: Preparative polyacrylamide gel electrophoresis of ^{14}C-labeled iodoacetate-treated zinc-free insulin: [iodoacetate]:[insulin], 20:1. Reaction time 18 hr in 0.2 M sodium acetate buffer (pH 5.6), containing 3.2 M urea. (○) Absorbance at 280 nm; (●) radioactivity.

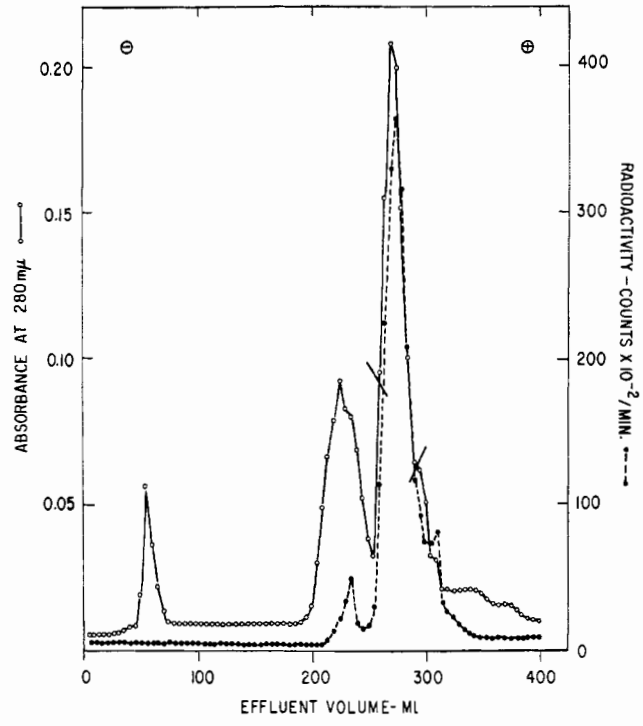

FIGURE 4: Preparative gel electrophoresis of the pooled fractions of the peak (between marks) of Figure 3. Experimental conditions as described in the legend of Figure 3.

results are shown in Figure 3. Most of the radioactivity corresponded to one of the three protein peaks. The samples between the two marker lines (effluent volume 250–290 ml) were pooled and precipitated with ether and the suspension was centrifuged at +4° for 15 min at 15,000*g*. The sediment was redissolved in 0.1 M sodium citrate buffer (pH 3.8) containing 4 M urea and subjected to a second preparative electrophoresis as described above. The results are depicted in Figure 4. Considerable additional purification was obtained. The pooled peak fractions from this gel (35 ml) were then precipitated and sedimented as already described. The precipitate was redissolved in 0.01 M Tris-Cl buffer in 4 M urea at pH 7.5 and subjected to chromatography on a DEAE-Sephadex A-25 column (1.8 × 22 cm) as described by Bromer and Chance (1967), as shown in Figure 5. The protein fractions were then eluted by a linear NaCl gradient (0–0.5 M) in the same buffer. The radioactive peak eluted between 0.3 and 0.4 M NaCl, the fractions were pooled, and the protein was precipitated with ether, lyophilized, and dried *in vacuo* under P_2O_5. The amount of protein recovered was 58 mg (yield 13.8%).

The purity of this material was ascertained by analytical disc gel electrophoresis. The pattern obtained in 4 M urea at pH 3.8 is shown in Figure 6, where the alkylated derivative is compared with the original insulin. The decreased cathodal migration of the essentially pure CM-insulin is readily apparent.

The purified CM-insulin was then subjected to the separation of the two peptide chains (see Materials and Methods). The amino acid composition of the B chain was determined by standard procedures. The result of the analysis is shown in Table I. It is evident that in the insulin derivative both histidyl residues are fully alkylated. The ratio of 3-*N*-carboxymethylhistidine to the 1-N isomer was approximately 6:1.

Biological Properties of CM-insulin. CM-insulin has been subjected to a standard radioimmunoassay and its potency in stimulating glucose oxidation and glycogen formation in the isolated rat diaphragm has also been investigated.

Insulin which has been subjected to the same manipulations

TABLE I: Amino Acid Composition of the B Chain of CM-insulin.

Amino Acid	Residues/Molecule	
	Native Insulin	CM-insulin
Lysine	1	1.0
Histidine	2	0
Arginine	1	1.1
Aspartic acid	1	1.0
Threonine	1	0.9
Serine	1	0.9
Glutamic acid	3	3.0
Proline	1	0.8
Glycine	3	3.0
Alanine	2	2.0
Half-cystine	2	1.7[a]
Valine	3	3.1
Leucine	4	4.0
Tyrosine	2	2.0
Phenylalanine	3	3.1
1-*N*-Carboxymethylhistidine	0	0.3
3-*N*-Carboxymethylhistidine	0	1.6

[a] Calculated as *S*-carboxymethylcysteine in the reduced and alkylated B chain.

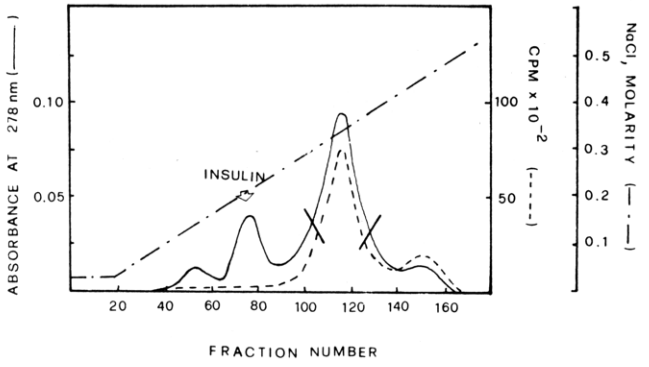

FIGURE 5: DEAE-Sephadex A-25 chromatography of CM-insulin from the pooled fractions of the second preparative electrophoresis. Elution with a linear NaCl gradient (0–0.5 M) in 0.01 M Tris-HCl buffer with 4 M urea (pH 7.5). (——) Absorbance at 278 nm; (- - - -) radioactivity; (—·—·—) NaCl molarity.

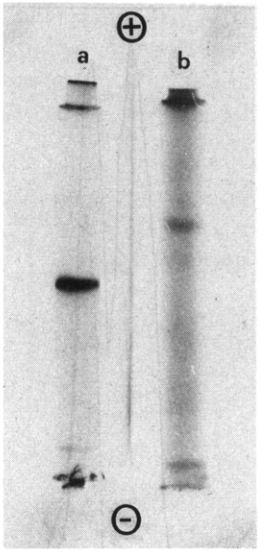

FIGURE 6: Analytical disc gel electrophoresis of 100 μg of zinc-free insulin (a) and purified CM-insulin (b). Conditions: 6% Cyanogum-41 in 0.1 M sodium citrate buffer with 4 M urea (pH 3.8). Cathodal migration for 90 min with 6 mA/tube.

of the derivative, except for the alkylation, was used as control. No significant differences have been found between the two molecules by standard radioimmunoassay, whereas a marked decrease of the ability to oxidize glucose as well as to incorporate glucose-*1*-^{14}C into glycogen was shown by the alkylated derivative, as reported in Table II.

Discussion

In a preceding paper (Covelli and Wolff, 1967) it has been shown that extensive iodination of zinc insulin at pH 8.5 led to the formation of a halogenated derivative in which, besides all the four tyrosyl residues, only *one* of the two histidines has reacted. However, when the same iodination was performed on zinc-free insulin both histidyl residues were readily available for iodination.

It has been recently demonstrated that zinc is coordinated to the N-3 imidazole nitrogens of the B^{10} histidyl residues of the insulin hexamer, which contains two atoms of zinc, and that this histidine plays an important role in the hexameric arrangement of zinc-insulin in solution (Blundell *et al.*, 1972). It seems likely, therefore, that the B^5 histidyl residue is the one that is readily available for iodination and that the reactivity of His-B^{10} toward iodine is restricted by zinc.

Under the conditions used here the carboxymethylation reaction is very much slower, and neither of the two histidyl residues appears to be readily available for alkylation when zinc is present in the insulin molecule. However, when zinc is removed, both histidines promptly react.

At present the reason for which in zinc-insulin one histidine, likely His-B^5, is readily available for iodination but not for N-alkylation is not clear. Factors such as the pH of reaction (5.6 for iodoacetate and 8.5 for I_2), the site of substitution (N-3 of N-1 for iodoacetate and C-4 for I_2) and the quaternary structure of the molecule could play a role in the relative resistance to carboxymethylation of the histidyl residues of zinc-insulin.

Since the region B^{10}–A^{8-10} of the molecule seems to represent an antigenic determinant (Arquilla and Stanford, 1972), it is interesting to note that CM-insulin possesses the same immunoreactivity than the native molecule, even though its biological activity is markedly lower than that of the native hormone (see Table II). It is possible, therefore, either that the chemical modification of histidines fails to alter immunoreactivity or that the antibody used in the standard radioimmunoassay is not very sensitive to this type of substitution.

TABLE II: Stimulation of $^{14}CO_2$ Production and Glucose-*1*-^{14}C Incorporation by Insulin and CM-insulin in the Rat Diaphragm.[a]

Compound	(Cpm/mg per hr) ± SEM $^{14}CO_2$ Produced	Glucose-*1*-^{14}C Incorp
None	428 ± 36 (6)	5,928 ± 641 (8)
Insulin (0.25 ng/ml)	977 ± 71 (8)	12,397 ± 1326 (12)
CM-insulin (0.25 ng/ml)	543 ± 48 (8)	8,128 ± 873 (12)

[a] The number of experimental samples is shown in parentheses.

References

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 236*, 1361.

Arquilla, E. R., and Stanford, J. (1972), *in* Insulin Action, Fritz, I. B., Ed., New York, N. Y., Academic Press, p 29.

Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., and Mercola, D. A. (1972), *Diabetes 21*, 492.

Bromer, W. W., and Chance, R. E. (1967), *Biochim. Biophys. Acta 133*, 219.

Covelli, I., and Wolff, J. (1966a), *Biochemistry 5*, 860.

Covelli, I., and Wolff, J. (1966b), *J. Biol. Chem. 241*, 4444.

Covelli, I., and Wolff, J. (1967), *J. Biol. Chem. 242*, 881.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 2413.

Dixon, G. H., and Wardlaw, A. C. (1960), *Nature (London) 188*, 721.

Field, G. B., Pastan, I., Johnson, P., and Herring, B. (1960), *J. Biol. Chem. 235*, 1863.

Flaschka, H. (1952), *Mikrochim. Acta 39*, 38.
Heinrikson, R., Stein, W. M., Crestfield, A. M., and Moore, S. (1965), *J. Biol. Chem. 240*, 2921.
Morgan, C. R., Sorenson, R. L., and Lazarow, A. (1964), *Diabetes 13*, 579.
Pastan, I., Roth, J., and Macchia, V. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1802.
Sluyterman, L. A. AE. (1955), *Biochim. Biophys. Acta 17*, 169.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Biochem. 30*, 1190.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Wolff, J., and Covelli, I. (1969), *Eur. J. Biochem. 9*, 371.

Chemical and Physical Properties of Serum Transferrins from Several Species†

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ABSTRACT: Bovine, rabbit, equine, and porcine serum transferrins have molecular weights of 77,300, 76,700, 79,100, and 76,400, respectively, in dilute aqueous solution as determined by sedimentation equilibrium centrifugation. The mercaptoethanol reduced proteins in 6 M guanidine hydrochloride possess molecular weights of 76,800, 77,400, 75,400, and 75,900, respectively. These results indicate that transferrin from all four species consists of a single polypeptide chain. Each transferrin binds two atoms of iron per molecule. Amino acid compositions of the four species of transferrins are similar but some significant differences exist. Comparison of the carbohydrate composition of these glycoproteins reveals similarities in the monosaccharides present but striking differences in their amounts. Each species contains mannose, galactose, glucosamine, sialic acid, and fucose, except equine in which fucose is absent. Bovine, rabbit, equine, and porcine transferrins contain a total number of 10–11, 18–19, 23, and 41–43 monosaccharide residues per molecule, respectively. It is postulated that this large variation in monosaccharide content is principally a result of differing numbers of heteropolysaccharide units per polypeptide chain: bovine contains one unit, rabbit and equine two, and porcine four, in analogy to chicken and human transferrins which have been shown to contain one and two units, respectively.

The transferrins represent a group of homologous nonheme iron binding glycoproteins found in blood serum of vertebrate animals, mammalian milk, and avian egg whites (Feeney and Komatsu, 1966). The function of serum transferrin is one of efficient transport of iron from the intestinal sites of absorption and the sites of hemoglobin breakdown to specific iron-requiring cells and to the various sites of storage (Fletcher and Huehns, 1968).

Serum transferrin from several species has been extensively studied with regard to amino acid composition, molecular weight, subunit structure, and iron binding capacity (Mann *et al.*, 1970; Palmour and Sutton, 1971; Greene and Feeney, 1968; Parker and Bearn, 1962). The amino acid compositions of human, monkey, rabbit, frog, turtle, and hagfish transferrin are quite similar but have significant differences. The molecular weight of human, rabbit, and frog transferrin is about 77,000, whereas turtle and hagfish have molecular weights of 92,000 and 44,000, respectively. Each of these transferrins consist of a single polypeptide chain as determined from molecular weight measurements in 6 M guanidine hydrochloride or 8 M urea on reduced and carboxymethylated proteins.

It is generally agreed that transferrin from different species possesses two iron binding sites, with the exception of hagfish transferrin which has one site (Palmour and Sutton, 1971). The relative affinity of the two sites has not been established. Warner and Weber (1953) and Davis *et al.* (1962) reported that the second ion bound to human transferrin had a greater affinity than the first. Contrary to these studies, Aasa *et al.* (1963) and Aisen *et al.* (1966) have reported that no difference exists in the affinity.

The carbohydrate structure of serum transferrins has been investigated in detail for two species, human and chicken. Striking differences exist between their carbohydrate moieties. Human transferrin has two identical heteropolysaccharide units composed of two residues of sialic acid, two residues of galactose, four residues of mannose, and four residues of *N*-acetylglucosamine (Jamieson, 1965; Jamieson *et al.*, 1971). In contrast chicken transferrin has most of its carbohydrate in a single heteropolysaccharide unit composed of either one or two residues of sialic acid, two residues of galactose, two residues of mannose, and three residues of *N*-acetylglucosamine (Williams, 1968). The carbohydrate units of both human and chicken transferrin are connected to the polypeptide chain through an *N*-glycosidic linkage to the amide of asparagine. Reports on the carbohydrate of transferrin from other species are limited to the sialic acid and hexosamine

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