removed by chromatography on silicic acid. Hexane-diethyl ether (25%) eluted the contaminant and hexane-diethyl ether (75%) eluted the glycerol ethers. The latter fraction had an infrared spectrum identical with a known glycerol ether, batyl alcohol, yield, 250 mg. It consumed 0.99 mole of periodate per mole of substance in 60 minutes and had an iodine number of 25. Samples of the glycerol ether were treated with periodate for 60 minutes, excess periodate destroyed, and extracted with chloroform. The water-soluble fraction gave, in approximately 75% yield, formaldehyde (as its dimedon derivative; m.p. 189°); the chloroform-soluble fraction readily formed a dinitrophenylhydrazone, and also a semicarbazone.

An α'-alkoxy-β-fatty acyl-α-glycerophosphorylethanolamine could be obtained from the "phosphatidylethanolamine" fraction through use of a modification of the deacylation procedure of Hübscher *et al.* (10). Briefly, this operation was carried out at 10° as follows: 2200 mg of the "phosphatidylethanolamine" fraction (Table I; 88 mg of P) in 20 ml of $CHCl_3$ were mixed with 200 ml of 0.5 N NaOH in methanol and allowed to react with stirring for 3 minutes. The pH was adjusted within 20 seconds to 6.85 by rapid addition of 6 N HCl. The reaction mixture was concentrated under reduced pressure at 30° to approximately 10 ml and then extracted twice with 10 volumes of $CHCl_3$. The turbid chloroform layer was washed once with 0.2 volume of water, cleared by storage at 4° for 3 to 4 hours. It was then evaporated to dryness under reduced pressure and dissolved to volume in chloroform. The latter fraction contained 82% of the added P (N:P, 0.98), whereas the water-soluble fraction contained 14% (N:P, 1.04; periodate uptake, 1.02 moles per mole of P). These results indicated that the deacylation had proceeded with primary attack on the diacyl phospholipids.[2] A more complete support for this conclusion was obtained through chromatography of the chloroform extract on silicic acid. Elution with chloroform removed the methyl esters (formed by methanolysis), chloroform-methanol, 4:1 (volume for volume) removed two small, colored peaks of low P content followed by a major peak containing 80% of the added P, (this was labeled Fraction IV) and finally chloroform-methanol, 1:4 (volume for volume) removed the component containing 15% of the added P (Fraction V). Nearly 96% of the P applied to the column was recovered, and analytical data on Fractions IV and V are recorded in Table I. Fraction IV is considered to be α'-alkoxy-β-fatty acyl-α-glycerophosphorylethanolamine, and Fraction V is predominantly a fatty acyl-free glycerol ether derivative, similar to that described by Carter *et al.* (5) and Svennerholm and Thorin (6). A short term acid hydrolysis (2 N HCl for 1.5 hours) of Fraction IV yielded fatty acids (unsaturated types only), free ethanolamine and glycerol ether phosphate in the expected amounts.

This study shows that α'-alkoxy phospholipids occur in bovine erythrocytes and are associated primarily with the "phosphatidylethanolamine" fraction. The biochemical relationship of these α'-alkoxy phospholipids to the vinyl ether plasmalogens and to the vinyl ether glycerides (11) provides a most interesting avenue of exploration.

[2] Although this reaction has been tried to a limited extent on the "phosphatidylethanolamine" fraction of human erythroctyes (which contains vinyl ether plasmalogens), no unique purification of the plasmalogens has resulted.

REFERENCES

1. Brante, G., *Acta Physiol. Scand.*, **18, Suppl.,** 63 (1949).
2. Dawson, R. M. C., *Biochem. J.*, **56,** 621 (1954); **75,** 45 (1960).
3. Edgar, G. W. F., *Acta Anat.*, **27,** 240 (1956).
4. Weiss, B., *J. Biol. Chem.*, **223,** 523 (1956).
5. Carter, H. E., Smith, D. B., and Jones, D. N., *J. Biol. Chem.*, **232,** 681 (1958).
6. Svennerholm, L., and Thorin, H., *Biochim. et Biophys. Acta*, **41,** 371 (1960).
7. Hanahan, D. J., Watts, R., and Pappajohn, D., *J. Lipid Research*, **1,** 421 (1960).
8. Norton, W. T., *Biochim. et Biophys. Acta*, **38,** 340 (1960).
9. Bevan, T. H., Brown, D. A., Gregory, G. I., and Malkin, T., *J. Chem. Soc.*, 127 (1953).
10. Hübscher, G., Hawthorne, J. N., and Kemp, P., *J. Lipid Research*, **1,** 433 (1960).
11. Eichberg, J., Gilbertson, J. R., and Karnovsky, M. L., *J. Biol. Chem.*, **236,** PC15 (1961).

Studies on Carboxypeptidase Digests of Human Hemoglobin

Eraldo Antonini, Jeffries Wyman, Romano Zito, Alessandro Rossi-Fanelli, and Antonio Caputo

From the Institute of Biological Chemistry, University of Rome, "Regina Elena" Institute for Cancer Research, Rome, Italy

(Received for publication, June 20, 1961)

We have observed profound changes in the oxygen affinity, the Bohr effect, and other characteristics of human hemoglobin, after digestion with carboxypeptidase A or B, or with both together.

The hydroxyl-terminal sequences of the α and β chains of human hemoglobin may be tentatively written as follows (1, 2):

α chain: -Thr-Ser-Lys-Tyr-Arg

β chain: -Ser-Ala-His-Lys-Tyr-His

We might thus expect carboxypeptidase A to act selectively on the β chains, carboxypeptidase B to act selectively on the α chains, and the joint action of the two enzymes together to exceed the sum of their actions separately. That these expectations are indeed fulfilled will be apparent from the results given in Table I. In no case does CPA[1] alone liberate any arginine, the hydroxyl-terminal residue of the α chains; nor does CPB alone liberate any histidine, the hydroxyl-terminal residue of the β chains. Apparently, CPA removes the last two residues, histidine and tyrosine, of the β chains, after which action nearly stops; similarly, CPB removes the last three residues, arginine, tyrosine, and lysine, of the α chains, after which it also comes to a stop.[2]

[1] The abbreviations used are: CPA, carboxypeptidase A; CPB, carboxypeptidase B.

[2] The results obtained on May 22 are to a degree an exception; the prolonged digestion with a high concentration of CPA led to values for histidine and tyrosine greater than 1, as well as to exceptionally high values for "other" residues; on the other hand, the usual 4-hour digestion with CPB failed to liberate the expected amount of tyrosine.

TABLE I

Results of digestion of native hemoglobin with carboxypeptidases at 30°, pH 8

After digestion, the solutions were extracted with trichloroacetic acid, and the extracts, after removal of the acid, were made 0.2 N in sodium citrate, pH 4. The amino acids were then determined by ion exchange chromatography (9). Controls were run by adding known amounts of free amino acids to the digests. Recoveries in all cases were better than 90%; the figures given in the tables are corrected for these recovery factors.

	Date*	Enzyme†	CPA/Hb	CPB/Hb	Digestion time	No. of amino acid residues liberated per αβ unit (MW 34,000)				
						His	Tyr	Arg	Lys	Others
			g		*min*					
CPA	Mar 3 (5)	W 1	1/20		90	0.48	0.45	0	0	0
					135	0.70	0.65	0	0	0
					195	0.93	0.88	0	0.06	0.28
	Apr 23 (14)	S 1	1/23		180	0.98	1.00	0	0.03	0.26
			+1/23		+180	1.01	1.04	0	0.08	0.59
	May 22	W 2	1/25		240					
			+1/25		+180					
			+1/25		+240	1.45	1.58	0	0.26	4.26
	June 2	W 2	1/25		240	0.81	0.81	0	0.11	1.47
CPB‡	Mar 18 (6)			1/135	150	0	0.93	0.96	0.84	0.21
	Mar 20 (9)			1/100	60	0	0.96	1.02	0.90	0.28
	Apr 23 (15)			1/215	180	0	0.99	1.01	0.97	0.27
				+1/215	+180	0	0.97	0.97	1.01	0.53
	May 22 (18)			1/100	240	0	0.16	0.83	0.23	0.52
	June 2 (21)			1/100	240	0	0.34	0.90	0.41	2.17
CPA + CPB	Apr 11 (12)	W 1	1/30	1/110	210	1.79	1.78	0.98	2.05	0.75
	Apr 23 (16)	S 1	1/23	1/215	180	1.11	1.90	lost	1.63	0.54
			+1/23	+1/215	+180	1.06	1.94	lost	1.80	1.65
	May 22 (19)	W 2	1/12.5	1/100	420	1.24	1.38	0.91	1.23	1.05
	June 2 (22)	W 2	1/25	1/90	240					
			+1/25	+1/90	+240	1.40	2.03	0.93	1.78	5.22

* Numbers identify digestion.

† W = Worthington; S = Sigma; numbers refer to lot.

‡ CPB was prepared from swine pancreas by the method of Folk et al. (8).

The results of the double digestion by CPA + CPB are less clear-cut. Although there is no doubt that under the joint action of the two enzymes two lysine residues are liberated per half molecule, in one experiment nearly two histidines (presumably the second being the fourth residue of the β chain) were liberated, whereas in another only one histidine was found. These inconsistencies, however, are relatively minor.

Each of the three digestions, whether with CPA, CPB, or CPA + CPB, gives a product which appears to be monodisperse in the ultracentrifuge and has a sedimentation constant essentially the same as that of normal hemoglobin (Table II). There is, thus, no indication of a change of molecular weight. Measurements with the Beckman model DK 1 recording spectrophotometer show that within the reproducibility of the tracings all three products have the same spectrum as normal hemoglobin in the visible, both in the oxygenated and deoxygenated form. All three digests have now been crystallized from ammonium sulfate at 0°. Experiments on the CPA and CPA + CPB products with moving boundary electrophoresis at pH 6.8 gave a single peak. We may, therefore, at least in the case of the better experiments, regard the end product of each type of digestion as a fairly definite modification of the hemoglobin molecule. We shall, for convenience, refer to the three products as HbCPA, HbCPB, and HbCP(A+B).

TABLE II

Sedimentation constants of native and modified hemoglobins

Compound	Solvent	pH	Concentration of solvent	$s_{20,w}$
			M	
Hb				4.40
HbCPA	NaCl	Neutral	0.03	4.60
HbCPB	Phosphate	7.0	0.05	4.30
HbCP(A + B)	Phosphate	7.0	0.02	4.44

Certain aspects of the physicochemical behavior of these modified hemoglobins have now been studied: (*a*) the oxygen equilibrium; (*b*) the kinetics of dissociation of the oxygen compounds; (*c*) the number of titratable —SH groups. Observations on acid and alkaline denaturation, heat stability, and titration curves have also been made.

All three modified hemoglobins (CPA, CPB, and CPA + CPB) show dramatic and highly characteristic changes in the oxygen

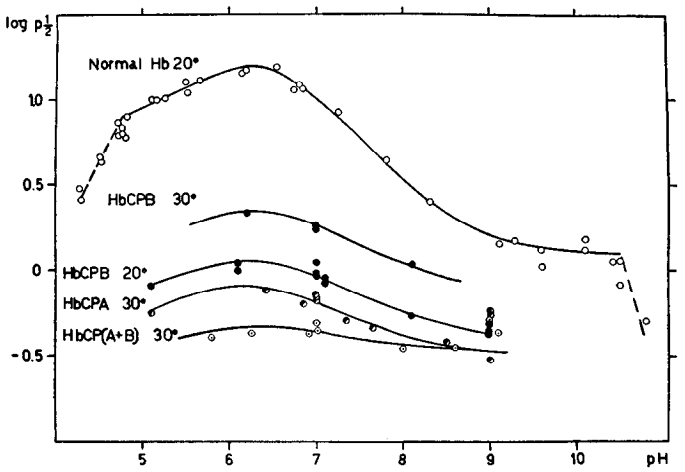

FIG. 1. Values of log $p_{\frac{1}{2}}$ as a function of pH for normal and digested hemoglobins. Hb concentration, ~ 3 g per liter. Buffers: 0.4 M acetate, pH 4.5 to 5.7; 0.2 M phosphate, pH 5.5 to 8.2; 0.2 phosphate-NaOH, 0.05 M borate, or 0.4 M glycine-NaOH, pH 8.2.

equilibrium; in each case a definite end point is approached as digestion proceeds. Indeed, the functional end point seems to be more definite and reproducible than the chemical one. The changes in the oxygen equilibrium, measured by methods previously reported (3), may be described conveniently and with sufficient accuracy in terms of the two parameters $p_{\frac{1}{2}}$ and n of the familiar empirical Hill equation

$$y = (p/p_{\frac{1}{2}})^n/[1 + (p/p_{\frac{1}{2}})^n]$$

Here, y is the fractional saturation of the hemoglobin with oxygen; p, the partial pressure of oxygen; and n, a dimensionless exponent representing in an over-all way the stabilizing interactions between the oxygen-binding sites; $p_{\frac{1}{2}}$ is the value of p at which $y = \frac{1}{2}$. At pH 7, the final value of $p_{\frac{1}{2}}$ for HbCPA is about $\frac{1}{30}$ that of normal hemoglobin ($\Delta \log p_{\frac{1}{2}} = -1.5$), and the value of n is unity as compared with 2.8 or 2.9 in normal hemoglobin. In fact, the molecule resembles myoglobin except that its oxygen affinity is about 3 times greater. At the same pH, HbCPB has a value of $p_{\frac{1}{2}}$ about 0.1 that of normal hemoglobin (about the same as myoglobin) but, in striking contrast to HbCPA, it retains a value of n only slightly less than that of normal hemoglobin, namely, about 2.7. In HbCPB therefore the interactions remain essentially unchanged. The double digestion with CPA and CPB gives rise to a form which, like HbCPA, has a value of n of exactly 1, but which shows an even higher oxygen affinity, $p_{\frac{1}{2}}$ at pH 7 being about 0.02 that of normal hemoglobin ($\Delta \log p_{\frac{1}{2}} = -1.7$). Whenever the effects of the digestion with CPA or CPA + CPB were followed in time, it was observed[3] that the drop of n occurred more rapidly than that of $p_{\frac{1}{2}}$. This suggests that the critical event as regards the drop of n in HbCPA and HbCP(A + B) may be simply the removal of the terminal histidine from the β chains. Similarly, the fact that in the experiment of May 22 digestion with CPB led to the full functional end point although only a small amount of tyrosine had been detached suggests that the critical event in this process is the removal of the terminal arginine from the α chains.

TABLE III

Values of kinetic dissociation constant

Hemoglobin concentration 0.5 g per liter; buffer at pH 7; 0.1 M phosphate buffer at pH 9.1; 0.05 M borate.

Substance	Values of k, sec^{-1}, at 21° ± 0.3	
	pH 7	pH 9.1
Hb	40	14
HbCPA	16	11
HbCPB	23	15
HbCP(A + B)	12	11

An extensive study was made of the Bohr effect, which may be described in terms of the relation between log $p_{\frac{1}{2}}$ and pH, provided the shapes of the oxygen equilibrium curves are independent of pH. Measurements were made at both 20° and 30°. For HbCPA and HbCP(A+B), it was found that the curves are strictly invariant in shape (with $n = 1$) for all changes of pH; so also (within the errors of measurement) are the curves for normal Hb, in accordance with what has been found previously for such other mammalian hemoglobins as have been studied.[4] For HbCPB, the curves are nearly, if not quite, invariant in shape, although there may be a slight tendency, doubtful in view of the scatter of the observations, for n to be less at pH 9 than at pH 7 (and also less at 30° than 20°). This invariance makes it possible to present the results of the study in terms of a graph of log $p_{\frac{1}{2}}$ as a function of pH (Fig. 1).

Fig. 1 shows that the Bohr effect is of the same magnitude in HbCPA as in HbCPB, notwithstanding the profound difference of n between the two, and is about $\frac{1}{3}$ as great as in normal hemoglobin. In HbCP(A+B) it is almost if not wholly lacking.

From the observed variation of log $p_{\frac{1}{2}}$ with temperature the following values of ΔH, the average value of the heat of dissociation of a single mole of oxygen, were calculated: HbCPA, −10,500; HbCPB, −12,500; HbCP(A+B), −12,000 cals. For native hemoglobin, the value (corrected for the heat of dissociation of the oxygen-linked protons) is about −12,000 (4).

Some kinetic measurements on the dissociation of oxygen from each of the three modified hemoglobins have now been made with a Gibson stopped flow apparatus (5), using dithionite as an oxygen absorber. In each case the reaction was found to be a strictly first order process over the observed range, from 95 to 20% saturation. Control measurements were also made on normal hemoglobin. Values of the dissociation constant obtained at pH 7 and pH 9 are as follows.

It will be seen that of the great increase of oxygen affinity observed at pH 7 for all three of the modified hemoglobins only a small part can be attributed to an effect on the dissociation constant; at pH 9, virtually none can be. The rather small effects of pH on k show that the equilibrium Bohr effect observed in HbCPA and HbCPB is considerably greater than can be explained in terms of a change in the kinetic dissociation constant alone. (Compare (6)).

We determined the number of free —SH groups present in HbCPA, HbCPB, HbCP(A+B), and native hemoglobin by

[3] In the early stages of the digestion, however, the values of n obtained from plots of log $[y/(1-y)]$ *versus* log p generally varied with the degree of oxygenation, tending to be higher at large values of y.

[4] In his most precise measurements, Roughton (7) finds evidence that the successive equilibrium constants of normal sheep hemoglobin are differently affected by pH.

spectrophotometric titration with *p*-mercuribenzoate, in a Beckman model DK1 instrument. In all four cases, the number of free —SH groups observed was 2 per molecule (68,000) to within ±5%.

We have also studied the rates of denaturation, by acid, alkali, and heat of native hemoglobin, and of the three modified forms. The results, which will be reported in detail later, show no striking differences. Likewise, the titration curves of the native and modified forms show no indication of the unmasking of any substantial number of titratable groups in the various digestion products.

Observations on Myoglobin—When human myoglobin is subjected to the same digestions with CPA and CPB as hemoglobin, there is, as would be expected, no change in n, which is already equal to unity. More striking is the fact that there is either no change at all, or at most only a slight change, in $p_{\frac{1}{2}}$ (50% or less). This is to be contrasted with the 30-fold decrease in $p_{\frac{1}{2}}$ which occurs in the case of hemoglobin. Although no quantitative amino acid determinations were made on the myoglobin digests, paper chromatography showed that in each case considerable quantities of amino acids had been liberated.

REFERENCES

1. Braunitzer, G., Hilschmann, N., Rudloff, V., Hilse, K., Liebold, B., and Müller, R., *Nature (London)*, **190**, 480 (1961).
2. Konigsberg, W., Guidotti, G., and Hill, R. J., *J. Biol. Chem.*, **236**, PC55 (1961).
3. Rossi-Fanelli, A., Antonini, E., and Caputo, A., *J. Biol. Chem.*, **236**, 391, 397 (1961).
4. Wyman, J., in M. L. Anson and J. T. Edsall (Editors), *Advances in protein chemistry, Vol. IV*, Academic Press, Inc., New York, 1948, p. 407.
5. Gibson, Q. H., in J. A. V. Butler and B. Katz (Editors), *Progress in biophysics and biophysical chemistry, Vol. 9*, Pergamon Press, London, 1959, p. 2.
6. Dalziel, K., and O'Brien, J. R. P., *Biochem J.*, **78**, 236 (1961).
7. Roughton, F. J. W., Conference on Hemoglobin, Publication 557, National Academy of Sciences, National Research Council, Washington, D.C., 1958.
8. Folk, J. E., and Gladner, J. A., *J. Biol. Chem.*, **231**, 379 (1958).
9. Moore, S. D., Spackman, D. H., and Stein, W. H., *Anal. Chem.*, **30**, 1185 (1958).

Inhibition of the Insulin Effect on Sugar Transport by *N*-Ethylmaleimide

E. Cadenas, H. Kaji, C. R. Park, and Howard Rasmussen

From the Department of Physiology, Vanderbilt University School of Medicine, Nashville 5, Tennessee, and the Rockefeller Institute, New York 21, New York

(Received for publication, July 10, 1961)

Although Stadie *et al.* (1, 2) have presented evidence for a binding of insulin to muscle tissue, the chemical nature of this attachment is not known. Recent studies on the binding of the neurohypophyseal hormones (vasotocin, vasopressin, and oxytocin) to the isolated amphibian urinary bladder (3, 4) and the mammalian kidney (5) have indicated that these hormones are probably bound to these tissues by means of a disulfide bond formed as a result of an interaction between a receptor sulfhydryl group and the disulfide bridge of the hormone. Because of the similarity between the intrachain disulfide-closed ring of insulin and the disulfide-closed ring of the neurohypophyseal hormones, a study was undertaken to determine whether or not insulin is also bound to tissues by a similar reaction. This report is the first phase of this study, and is concerned with the effect of *N*-ethylmaleimide, a sulfhydryl blocking reagent, upon the binding and activity of insulin on the isolated perfused rat heart.

Hearts from Sprague-Dawley rats, fasted about 18 hours, were perfused by the apparatus and methods described previously (6). In a typical experiment, the hearts were first washed free of blood by perfusion with Krebs-Ringer bicarbonate buffer for 2 minutes at 37°. The perfusion was then shifted for 30 seconds to buffer containing EM[1] in a concentration of 1×10^{-3} M. This was then washed out by perfusion with plain buffer for 2.5 minutes, a time sufficient to remove more than 95% of a substance the size of EM from the extracellular fluid (6). Perfusion was then begun with a medium containing the test sugar either with or without insulin. Controls were subjected to a preliminary perfusion without EM.

This exposure to EM usually caused a slight weakening in the force of contraction and a slowing of the heart rate, but did not produce any change in perfusion flow or pressure. Perfusion with EM for longer periods of time resulted in a progressive loss of mechanical activity and increased accumulation of intracellular glucose.

The effects of pretreatment with EM were studied by three different methods for estimating the transport of sugar (6, 7). The results are recorded in Table I.

In Experiment A, transport was estimated by the rate of utilization of glucose from a glucose-poor medium (50 mg/100 ml), a circumstance in which transport becomes the rate-limiting step for utilization (6). Pretreatment with EM caused an 80% inhibition of the insulin-accelerated utilization but had no significant effect upon utilization in the absence of insulin, nor did it cause the intracellular accumulation of glucose under any condition.

With a high concentration of glucose in the medium (800 mg/100 ml) higher rates of transport can be obtained, and with insulin present, substantial quantities of glucose accumulate inside the cell owing to the rapid penetration of the sugar relative to the rate of intracellular phosphorylation (6). This insulin-dependent intracellular accumulation of glucose was virtually abolished by pretreatment with EM (Experiment B, Table I), indicating a substantial reduction in transport rate.

L-Arabinose is transported by the same system as glucose (7), but is not phosphorylated. Its intracellular accumulation is a third method of estimating insulin-dependent transport. As shown in Experiment C, Table I, pretreatment with EM greatly reduced transport of L-arabinose in the presence of insulin.

To test whether exposure to insulin would prevent the subsequent effect of EM, rats were given intravenous injections of about 60 μg of insulin per 100 g of weight 10 minutes before the hearts were removed. The tissue was then exposed to EM and insulin. As seen in Experiment D, Table I, no significant inhibition of transport was produced by EM under these conditions.

[1] The abbreviation used is: EM, *N*-ethylmaleimide.