Dialysis Studies. IX. On the Conformational Stability of Glucagon, Adrenocorticotropic Hormone, and Similar Peptides*

L. C. Craig, J. D. Fisher, and T. P. King

ABSTRACT: The membrane diffusion behavior of a number of intermediate sized polypeptides, adrenocorticotropic hormone, glucagon, parathyroid hormone, and the like, whose shape is not restricted by covalent cross-links, has been investigated. The rates of diffusion were found to be strongly influenced by small alterations in the solvent, particularly ionic strength and temperature.

The behavior is consistent with the view that the polypeptides assume the shape of random coils whose effective conformation is strongly influenced by the solvent environment. This could be of importance in *in vivo* bioassays where different routes of administration are being compared.

In earlier papers (Craig *et al.*, 1957, 1958; Craig and Konigsberg, 1961) data have been presented to show that a simple dialysis technique with cellophane membranes can be employed in such a way that it offers a very useful test for homogeneity with respect to size. Attempts to improve further the discriminatory features of the procedure have raised many questions concerning the nature of the membrane, the influence of the solvent, the individual behavior of the solute, and the way all three factors interact to influence the rate of diffusion through the membrane. Information concerning each factor has been derived by studying the influence of various conditions on the relative rates of dialysis of different types and sizes of solutes, sugars (Craig and Pulley, 1962), amino acids (Craig and Ansevin, 1963), peptides (Craig *et al.*, 1964), proteins (Craig *et al.*, 1958), and the like. Many of the solutes have had ionizable groups but others have contained none. The question of the shape of the molecule and its tendency to associate or aggregate has been of primary interest. In many cases, the solutes were studied concomitantly in the ultracentrifuge.

From the standpoint of the shape factor, and also the aggregation tendency, it seemed of interest to make a study of large polypeptides with no covalent cross-links to restrict the shape of the molecule. It might be suspected that the effective shape of such molecules would be influenced to a much greater degree by the solvent than the more rigidly held proteins.

Unfortunately, model substances of this type are not readily available. Possibilities include the parathyroid hormones (Rasmussen and Craig, 1962), adrenocorticotropic hormones (Li, 1956), synthetic ACTH[1] fragments (Li *et al.*, 1961; Hofmann *et al.*, 1962; Kappeler and Schwyzer, 1961), glucagon (Bromer *et al.*, 1957), and the A and B chains from insulin (Craig *et al.*, 1961). This paper will report our experience thus far with these substances. Some of the data obtained with parathyroid hormones has been published previously.

Materials and Methods

The dialysis experiments were made in the thin-film dialysis cells described in an earlier paper (Craig and Konigsberg, 1961). The membranes used were all from Visking cellophane tubing modified by mechanical stretching or by controlled acetylation to give a suitable pore size. The pore size was chosen to give relatively high selectivities, ones which provided 50% escape times of at least 1 hour.

The glucagon sample was a gift from Dr. Otto Behrens of the Eli Lilly Co. The tricosapeptide (Hofmann *et al.*, 1962) was a gift from Dr. Klaus Hofmannn of the University of Pittsburgh. The porcine ACTH preparation was a gift from Dr. E. E. Hays of Riker Laboratories, Inc. The lipid-mobilizing hormone (Rudman *et al.*, 1962) was from a preparation fractionated by Dr. Howard Rasmussen using gel filtration. We wish to thank each of the above named for the opportunity to study the various materials.

The A and B chains from insulin were obtained by oxidizing beef insulin with performic acid (Craig *et al.*, 1961) and separating by countercurrent distribution. They were of satisfactory purity by amino acid analysis.

The dialysis results obtained with the samples of glucagon, tricosapeptide, and the lipid-mobilizing hormone were all consistent with the presence of a single molecular size in the preparation. Accordingly, no attempt was made to fractionate them further. The result obtained, however, with the crude ACTH preparation indicated the presence of more than one molecular size. It was therefore extensively fractionated by countercurrent distribution. The sample had been

* From the Laboratories of the Rockefeller Institute and Armour Pharmaceutical Company. *Received October 5, 1964.* This study was supported in part by a grant (AM 02493-06) from the U.S. Public Health Service.

[1] Abbreviation used in this work: ACTH, adrenocorticotropic hormone.

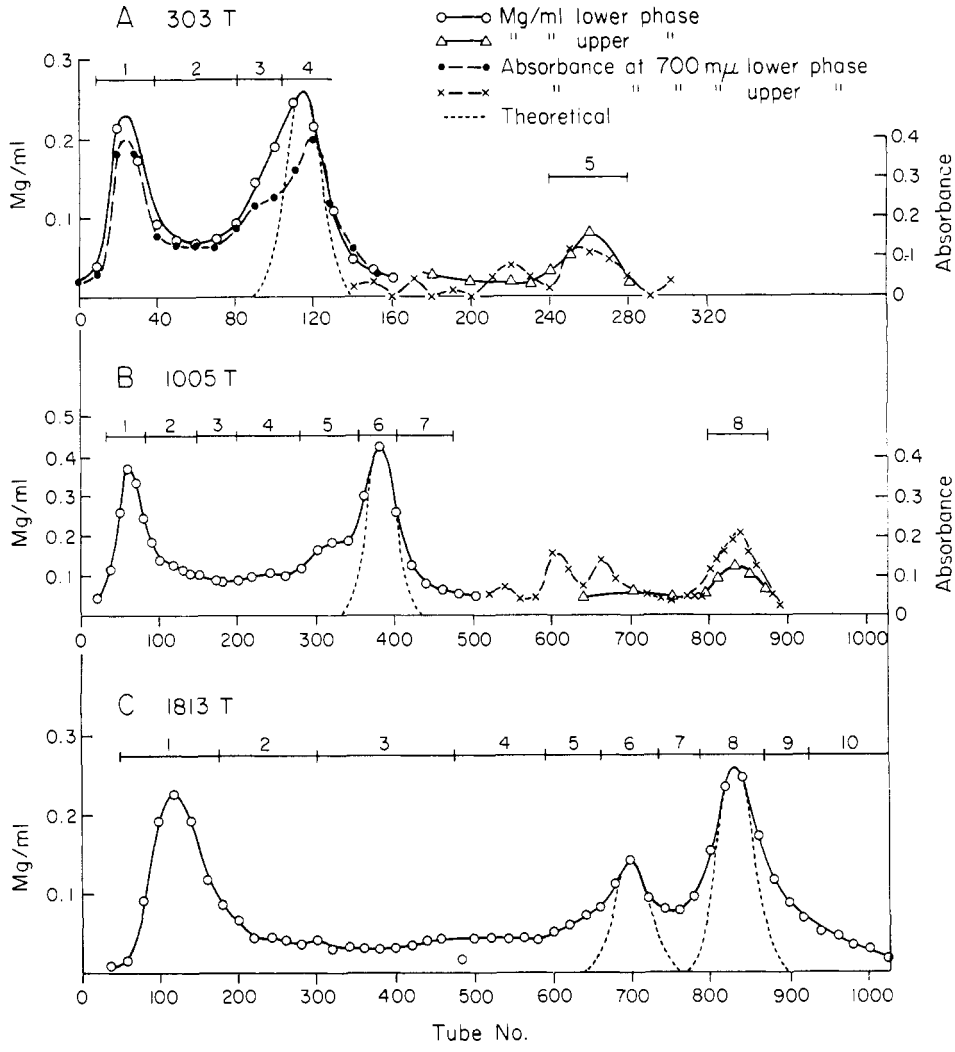

FIGURE 1: Countercurrent distribution patterns of a partially purified sample of porcine ACTH. The bars and numbers above the curves designate the various cuts taken.

obtained by Dr. Hays from Nordic Biochemicals, Ltd. It was considered to be a sample of high activity since an assay of 139.2 IU/mg (subcutaneous) had been found by the ascorbic acid depletion method. No claim was made for chemical purity.

The countercurrent distribution equipment used in fractionating the sample of ACTH was of the automatic design developed in this laboratory (Craig and King, 1958). The particular train employed contained 1020 tubes each with 5/3 ml capacity. The system was one found most effective for fractionating the parathyroid hormones (Rasmussen and Craig, 1962). It was made from 1-butanol–pyridine–ammonium acetate buffer (12.5:5:4). The ammonium acetate buffer was 0.1% acetic acid, 0.15 N ammonium acetate.

Several runs were made with different numbers of transfers and therefore of different times of exposure to the fractionating conditions. Within the limits of accuracy of the assay used we could not detect loss of activity even during the most prolonged treatment.

The active material was recovered from the solutions in the cuts from the train by concentration first under vacuum from a water aspirator in the rotatory evaporator and finally under vacuum from an oil pump with the residue flask held at 30°. When the ammonium acetate residue began to crystallize, 1-butanol saturated with water was added and the concentration repeated until the volatile salt was removed. The butanol appeared to be effective in helping to carry the ammonium acetate over into the condenser, which was cooled in dry ice and acetone. Finally, the residue was dissolved in 0.01 N acetic acid and lyophilized. Distribution patterns of the various runs are given in Figure 1. Analysis was made by residue weight (King and Craig, 1962).

Biological assays were made at the Armour Pharmaceutical Co. on samples sent from the Rockefeller Institute at three different times. Only materials from the second distribution, Figure 1B, were assayed utilizing the Sayers' assay procedure under conditions specified

TABLE I: Activities of Countercurrent Distribution Fractions of ACTH (μ/mg).

	Cut 1	Cut 2	Cut 4	Cut 5	Cut 6	Cut 7	Crude	Armour Sample[a]
Intravenous		1.5		35	60		40	
				21	73.8		42.5	
Average				28	66.9		41.2	
Subcutaneous	10	5	32.5 ± 3.8	35.6 ± 4	139.5 ± 12	89.0 ± 9	51.6 ± 4	49.5 ± 7.0
				42.0 ± 6.6	169.5 ± 39		63.6 ± 12	
				49.5	142 ± 22		79.6 ± 16.8	
				50.4 ± 5.2	141 ± 16.5		72.0 ± 7.8	
							76.2 ± 7.2	
Average				44.4	148		68.6	
				Wt mean = 42.8	Wt mean = 142		Wt mean = 64.0	
				P 95 = 37.3–49.0	P 95 = 126–160		P 95 = 58.0–70.7	
Subcutaneous/ Intravenous				1.6	2.2		1.66	

[a] Armour sample No. 907-098-A4, given to one of us (Craig) several years ago.

by the U.S. Pharmacopoeia XVI. Both the intravenous and subcutaneous routes of administration were employed. The data are presented in Table I.

Since considerable activity was found in cuts 4, 5, 6, and 7 from Figure 1B, samples from each of these were hydrolyzed in 6 N HCl at 108° for 24 hours and amino acid analyses were made in the Spinco recording amino acid analyzer by the Spackman-Stein-Moore procedure (Spackman *et al.*, 1958). The data are given in Table II.

Results and Discussion

Early in our studies with the thin-film dialysis method (Craig *et al.*, 1958), it was noted that the rates of dialysis of different proteins were influenced to different degrees by varying the *p*H, by addition of urea and particularly by addition of salt even in low concentration. This was interpreted as indicating that the shape of the proteins was being changed to some degree by the change in environment. However, it was not known at that time how much of the change in dialysis rate was due to change in the membrane structure or whether the presence of possible fixed charges on the membrane could play a role. Since that time a considerable accumulation of data has shown that (1) the effect of fixed negative charges on the membrane is indeed weak (Craig and Ansevin, 1963) and apparently negligible; (2) the effective pore size is changed only slightly by changes in ionic strength, *p*H, and urea; and (3) barring adsorption effects (Craig and Ansevin, 1963), 50% escape times can be compared reliably to give a type of information similar to that derived by comparison of diffusion coefficients. Because of the frictional effects of membranes adjusted (Craig and Konigsberg, 1961) or chosen so that their pores will barely permit diffusion, the differences noted in free diffusion are considerably amplified and relatively small effects may be detected (Craig and Pulley, 1962). With the foregoing information at hand, it seemed a particularly appropriate time to investigate the dialysis behavior of relatively long peptides devoid of covalent bonds such as disulfide linkages which restrict the shape of the molecule.

A concomitant study of shorter peptides containing up to 18 amino acid residues has been made (Craig *et al.*, 1964), including angiotensin and a number of its synthetic analogs, oxytocin, vasopressin, and synthetic analogs, and with the tryptic peptides obtained from the α and β chains of hemoglobin (Hill and Konigsberg, 1960; Guidotti *et al.*, 1962). In both the angiotensin and hemoglobin peptide series, peptides were found whose rates of dialysis were only slightly if at all influenced by the addition of ammonium acetate. The salt effect could be correlated with the relative positions of the acid and basic groups on the chain. Those peptides showing no salt effect generally had a positive and negative charge in close proximity at one end so that interaction would not cause the chain to bend significantly.

Urea at a concentration of 6 M markedly retarded the rate of diffusion of nearly all the peptides, although not to the same degree in all cases. Glucagon was an exception as will be seen later on. That this was not an effect on the membrane was shown by the fact that bacitracin, a cyclic dodecapeptide, gave a dialysis rate in 6 M urea which was very close to that obtained in water or 0.01 N acetic acid. With this peptide the rate also was not influenced by ammonium acetate.

When a sample of porcine ACTH of satisfactory purity was obtained, it was found to dialyze through a

TABLE II: Results from Amino Acid Analysis of ACTH Fractions.[a]

Amino Acid	Acid Hydrolysis			Alkaline Hydrolysis			
	Cut 5	Cut 6	Theory	Cut 4	Cut 5	Cut 6	Cut 7
Lysine	3.72	4.04	4				
Histidine	0.99	0.96	1				
Ammonia		1.72	1				
Arginine	2.8	2.91	3				
Aspartic acid	2.09	2.02	2	2.77	2.11	1.98	2.1
Methionine sulfoxide			0	0.28	0.26	0.04	0.02
Threonine	0.18	0.13	0	0	0	0.12	0.06
Serine	1.46	1.75	2	0.67	0.63	0.6	0.62
Glutamic acid	4.6	5.00	5	4.91	4.75	4.9	4.68
Proline	3.85	4.08	4	4.24	3.76	4.1	3.5
Glycine	2.82	2.88	3	4.96	3.83	3.78	3.9
Alanine	2.58	2.90	3	3.12	2.95	3.1	3.2
Half-cystine	0	0	0				
Valine	2.6	2.84	3	1.88	2.16	2.25	2.16
Methionine	0.91	1.00	1	0.63	0.66	0.92	0.71
Isoleucine	0.13	0.05	0	0.10	0	0	0
Leucine	2	2	2	2	2	2	2
Tyrosine	1.55	1.84	2	1.33	1.3	1.7	1.4
Phenylalanine	2.4	2.8	3	1.92	2.47	2.88	2.54

[a] Expressed as number of residues relative to leucine taken as 2.

membrane in an ideal way with 0.01 N acetic acid as the solvent (Figure 2a, curve 1). The rate when compared to that of other solutes of similar size was entirely consistent with the thesis that the hormone can form a closely packed monomeric molecule whose effective shape does not diverge too greatly from that of a sphere. This result was unexpected in the light of the data from the ultracentrifuge published recently by Squire and Li (1961). They found a sample of bovine ACTH to associate strongly in ammonium formate buffer at a *p*H not far from that which 0.01 N acetic acid would provide. The association was repressed in HCl-KCl, *p*H 1.3, ionic strength 0.200—a result to be expected from the earlier work of Brown *et al.* (1956) with porcine ACTH in 0.05 M KCl–0.05 M HCl. Diffusion coefficients indicated a frictional ratio of 1.46 from which with certain assumptions an axial ratio of 7:1 could be calculated.

When the rate of escape of our porcine ACTH was determined in the calibrated membrane with HCl-KCl, *p*H 1.36, ionic strength 0.200 as the solvent, curve 2 (Figure 2a) was obtained. The rate here was retarded 6-fold over that in 0.01 N acetic acid. The difference is not caused by the lower *p*H since a similar retardation was also noted when 0.01 N acetic acid was made 0.2 M with respect to NaCl. The simplest explanation is that a shift in conformation or expansion was produced by the salt.

When the rate of escape of the porcine ACTH was determined in 0.2 M ammonium acetate–0.3 M acetic acid buffer, curve 3, the 50% escape time was found to be even longer than in the sodium chloride solution. It was suspected from the data of Squire and Li that this was owing to association. However, Dr. D. Yphantis found the material to be almost entirely monomeric in equilibrium sedimentation studies in this buffer. The effect of 6 M urea, curve 4, was in the same direction but not as large as was found with ammonium acetate.

If the effective diffusional volume of the solute has undergone a change caused by the salt, the magnitude of the effect noted should be a function of the membrane selectivity which in turn is related to the critical size of the pores (Craig *et al.*, 1957). Therefore rates were compared in an unstretched membrane, one that gave a half-escape time of 0.37 hour for ACTH in 0.01 N acetic acid. The half-escape time in the ammonium acetate buffer was 2.1 hours, a 5.7-fold shift instead of the 14-fold shift found in the tighter-stretched membrane.

Optical rotatory dispersion measurements were made on the sample of ACTH. In 0.01 N acetic acid, a 0.425% solution of the hormone gave values from which the well-known Lowry plot could be constructed with a satisfactory fit. The wavelength covered ranged from 650 to 360 mμ. It was not possible to extend the readings below 360 mμ because of excessive absorption of the light. Extrapolation of the straight line gave a λ_c value of 238 mμ; $[\alpha]^{25°}_{600\ m\mu} = -64°$; $[\alpha]^{25°}_{400\ m\mu} = -180°$. These data are consistent with those expected from a random coil.

When the solvent was changed to the 0.2 M ammonium acetate–0.3 N acetic acid buffer, *p*H 4.3, a satisfac-

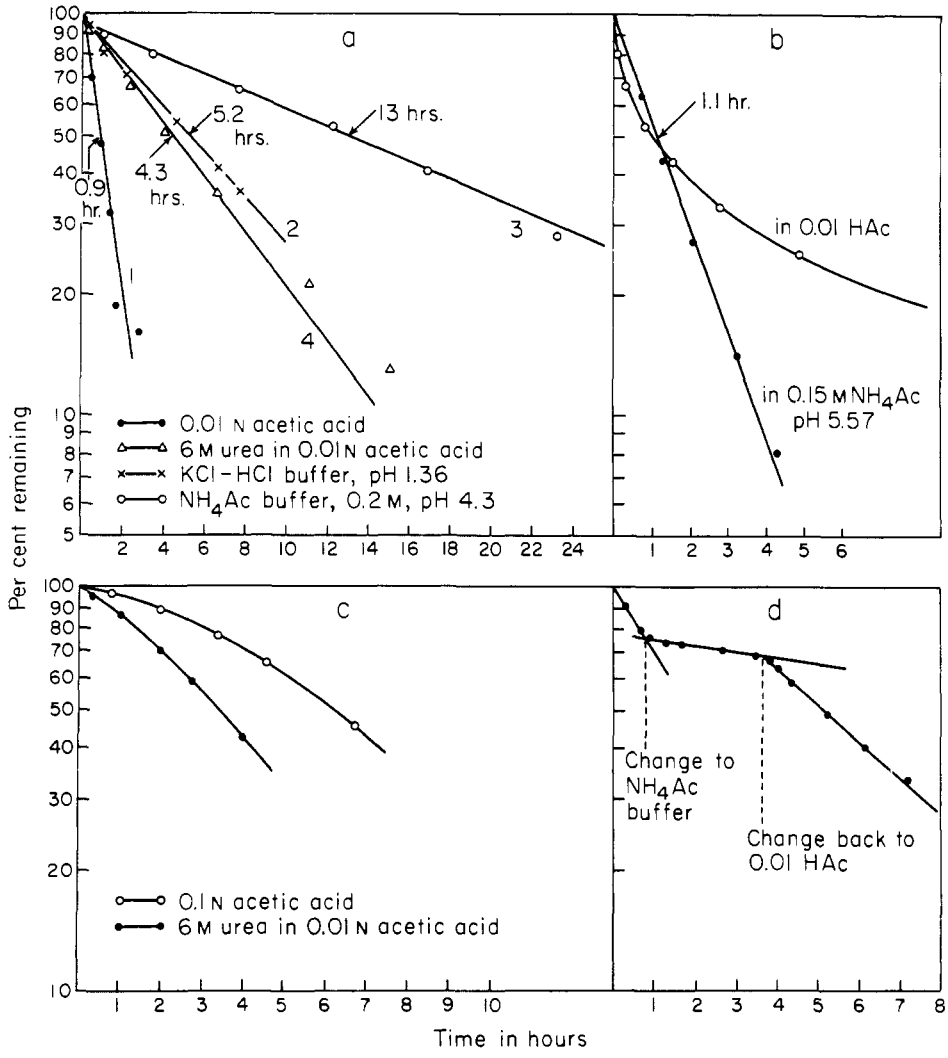

FIGURE 2: Membrane diffusion patterns of various peptides. (a) ACTH (cut 8 from Figure 1c); (b) the A chain from oxidized insulin; (c) glucagon; (d) glucagon.

tory linear Lowry plot was again obtained but with a small shift in the values, $\lambda_c = 229$; $[\alpha]^{25°}_{600\ m\mu} = -67°$; $[\alpha]^{25°}_{400\ m\mu} = -183°$. It would thus appear that the striking shift in conformation indicated by the dialysis to result when the solvent was changed from 0.01 N acetic acid to the acetate buffer has little effect on the optical rotation. This result was not unexpected since ACTH is not considered to have an ordered secondary structure. All of its available hydrogens are rapidly replaced in deuterium exchange experiments (Li, 1962).

Parathyroid hormone C (Rasmussen and Craig, 1962) was found to have properties very similar to those of ACTH. It did not fractionate well by most chromatographic procedures but could be isolated by countercurrent distribution in the same system used here for the purification of ACTH. It is nearly twice the size of ACTH and also has no covalent cross-linking bonds. It shows a similar striking change in dialysis rate when salt is added to the solvent. The shift in conformation is readily seen in its behavior in gel filtration. Sephadex G-25 provides a suitable gel structure if 0.01 N acetic acid is the solvent but G-50 is required if the solvent is the 0.2 M ammonium acetate–0.3 N acetic acid buffer. This correlation gives a strong suggestion of a way to make Sephadex more selective, a suggestion effectively used in parathyroid hormone fractionation (Rasmussen and Craig, 1962).

Of the peptides studied in this series all showed the same type of strong shift with the addition of ammonium acetate except the A chain of insulin. As can be seen from Figure 2b, it does not give a straight-line escape curve in 0.01 N acetic acid but is strongly concentration dependent. With the addition of ammonium acetate, however, ideal behavior is observed. This result seems to be due to the fact that the A chain has a single N-terminal basic group but seven acid groups (four sulfonic and three carboxyl) distributed along the chain. The increased ionic strength reduces the mutual repulsion of the negative charges and apparently permits

TABLE III: Comparative 50% Escape Times of Various Peptides.

Peptide	No. of Residues	Mol. Wt.	0.01 N HAc (25°)	0.2 N NH_4Ac (25°)	0.01 N HAc (40°)	0.2 N NH_4Ac (40°)
Bacitracin	12	1422	0.42			
Insulin A chain	21	2532		17		11
Synthetic tricosapeptide	23	2700	1.17	11	0.43	8.1
Glucagon	29	3550	3.5		1.15	24
Insulin B chain	30	3600	8	Not sol.	4.6	Not sol.
ACTH	39	4567	4.15		2	

a closer approach to a compact random coil. A similar result (Goldstein and Craig, 1960) has been noted with s-RNA.

Glucagon showed an interesting behavior. It had barely sufficient solubility for study in 0.01 N acetic acid and a little excess acetic acid of greater strength was required in the initial charge. The excess passed through the membrane in the first few minutes and should not be important in the escape curve. The escape curve (Figure 2c) was found to have a negative curvature. When 6 M urea–0.01 N acetic acid was the solvent there was no difficulty with solubility, a nearer approach to a straight line was obtained and the rate was faster than without the urea. Since urea usually slows the rate of dialysis by permitting a more expanded conformation it would seem likely that this hormone in 0.01 N acetic acid may not be completely dissociated to the monomer but is dissociated when urea is added. This point could not be reliably checked in the ultracentrifuge since the ionic strength needed resulted in too low a solubility. In 0.1 N acetic acid glucagon gave a straight line for the escape pattern but the 50% escape time was 1.6-fold that in 0.01 N acetic acid indicating a more extended conformation.

The effect of ammonium acetate on glucagon is strikingly shown in Figure 2d where the diffusion was started in 0.1 N acetic acid, but after the second diffusate was removed the outside solution was changed to 0.1 N acetic acid made 0.02 M with respect to ammonium acetate. This dramatically slowed the rate of dialysis. After continuing with this buffer until five diffusates had been collected the outside again was changed to 0.1 N acetic acid. Almost immediately the rate of dialysis increased and continued to give a straight line whose slope, however, did not return to the initial slope. This could be taken as an indication that the conformation did not quite return to the original average. Ammonium ion even in 0.002 M strength slowed the rate of dialysis 2.4-fold.

Table III gives comparative diffusion data with a number of peptides, all obtained with the same calibrated membrane. The tricosapeptide considered to have full ACTH activity (Hofmann *et al.*, 1962), glucagon, and ACTH (as previously discussed with a more porous membrane) all showed strikingly reduced dialysis rates when ammonium acetate was added. The B chain from insulin in 0.01 N acetic acid shows too slow a rate for the monomer and almost certainly diffuses as the dimer or trimer. Attempts to study it in the ultracentrifuge were hampered by solubility difficulties due to salting out but strong association was indicated. From the literature (Leach and Scheraga, 1958) it is known to be associated.

The temperature coefficients derived from the data in Table III are of interest. An ideal solute should dialyze 1.45 times as fast at 40° as at 25° owing to the higher temperature and lower viscosity. ACTH dialyzes 2.1 times as fast compared to 2.7 for the tricosapeptide. This can be interpreted as an indication of greater conformational stability with the larger peptide. Actually the difference between the two is probably greater than these figures indicate because the comparative rates with ACTH fall within a more selective range of pore sizes.

The tricosapeptide showed quite a different temperature coefficient in the ammonium acetate buffer. Here it diffused only 1.36 times as fast at the higher temperature, not far from ideal behavior.

The sample of lipid-mobilizing hormone dialyzed as if it had a molecular size very similar to that of ACTH. As with ACTH the rate of diffusion was strikingly reduced by ammonium acetate.

When all the data available for this type of peptide are considered it would appear that their behavior is best explained by some type of mobile equilibrium involving many different shapes. The statistical average of these forms, influenced in part by the interaction between individual molecules, determines the diffusional size or activity. This activity is influenced strongly by the solvent, the *p*H, the temperature, and undoubtedly to a certain degree by the amino acid sequence.

The distribution behavior of the ACTH sample also may well be influenced by the conformational stability of the molecule. It would seem from a comparison of the patterns in A, B, and C of Figure 1 that the bands from which cuts 4, 6, and 8 in pattern C are taken are not sharpening up as much as they should with the increasing numbers of transfers. This of course is an indication of a dispersion of molecular forms. The amino acid

analytical data discussed in following paragraphs are in line with the theory of a family of closely related isomers.

It would be expected from the foregoing discussion that, during the countercurrent distribution run, the average conformation in the organic phase would be very different from that in the aqueous phase. Therefore, true equilibration could well require a more rapid adjustment of conformational form than the conformational stability will permit. A broadened band could well result from this state of affairs depending on the overall balance and the concentration range covered. A band more narrow than the theoretical might also be expected in certain systems as in fact has been observed with insulin, ACTH, and serum albumin (Hausmann and Craig, 1958).

A further consequence of conformational stability and metastability may have to do with the biological assays of closely related polypeptides, where rates of diffusion and rates of enzymatic destruction are always involved. For example in Table I the biological assays of various cuts from Figure 1B are compared by two different routes of administration, intravenous and subcutaneous. Apparently any sample of ACTH is rapidly destroyed or inactivated by enzymes when it is injected into the blood stream, since the effect registers and then drops off in a matter of minutes (Sydnor and Sayers, 1953). Obviously the rates of absorption and inactivation could play a very important role and both of these can be related to the conformational stability.

The tricosapeptide of Hofmann and collaborators (Hofmann *et al.*, 1962) has been reported to give 100 μ/mg in the intravenous test but only 38 in the subcutaneous test. The subcutaneous/intravenous ratio is thus roughly 0.3 as compared to the finding in Table I of 2.2 for the naturally occurring hormone. Our membrane experiments seem to indicate that the smaller tricosapeptide may have less conformational stability than the 39 amino acid hormone.

Irrespective of the foregoing interesting possibilities it is interesting to ask what the chemical differences can be in the various biologically active cuts from Figure 1B. Amino acid analyses of these cuts were made in the Spinco amino acid analyzer according to the procedure of Spackman *et al.* (1958). Only cuts 4, 5, 6, and 7 show activity. From Table II only cut 6 is completely satisfactory from an analytical standpoint in that the amino acid residues account satisfactorily for the entire weight of the sample. Cut 5 was not as good. A test for carbohydrate material by the orcinol test (Tsugita and Akabori, 1959) in each of the active fractions was essentially negative.

It was thought that cuts 4 and 5 could arise from oxidation of the methionine residue. Accordingly, samples were hydrolyzed in alkali according to the procedure of Neumann *et al.* (1962). Amino acid analysis showed an appreciable content of methionine sulfoxide, 0.28 and 0.26, respectively, in cuts 4 and 5. The total of the methionine and methionine sulfoxide content agrees with that expected for 1 mole of methionine as was found by acid hydrolysis for fraction 5. This raises the question as to whether this particular methionine sulfoxide can revert to methionine even in alkaline hydrolysis. A second explanation could be based on the possibility that the hormone in the countercurrent distribution system is present as a dissociable dimer or trimer. Cut 5 then might result from a trimer containing 2 moles of the hormone and 1 mole of the methionine sulfoxide hormone. With either explanation further investigation is needed.

The fractionation results of the ACTH sample are similar to those recently reported by De Jager *et al.* (1963) using cellulose ion-exchange chromatography. However, their fractions showed inhomogeneity by paper electrophoresis. The hormone recovered from cut 6 of Figure 1B showed only a single zone by starch-gel electrophoresis. This contrasts with the experience of Barrett *et al.* (1962). The data obtained including the excellent amino acid analysis give strong support for the purity of the main fraction obtained by countercurrent distribution.

References

Barrett, R. J., Friesen, H., and Astwood, E. B. (1962), *J. Biol. Chem. 237*, 432.

Bromer, W. W., Sinn, L. G., and Behrens, O. K. (1957), *J. Am. Chem. Soc. 79*, 2807.

Brown, R. A., Davies, M., Englert, M., and Cox, H. R. (1956), *J. Am. Chem. Soc. 78*, 5077.

Craig, L. C., and Ansevin, A. (1963), *Biochemistry 2*, 1268.

Craig, L. C., Harfenist, E. J., and Paladini, A. (1964), *Biochemistry 3*, 764.

Craig, L. C., and King, T. P. (1958), *Federation Proc. 17*, 1126.

Craig, L. C., King, T. P., and Stracher, A. (1957), *J. Am. Chem. Soc. 79*, 3729.

Craig, L. C., and Konigsberg, W. (1961), *J. Phys. Chem. 65*, 166.

Craig, L. C., Konigsberg, W., and King, T. P. (1961), *Biochem. Prepn. 8*, 70.

Craig, L. C., Konigsberg, W., Stracher, A., and King, T. P. (1958), *Symp. Protein Struct. Paris, 1957*, 104.

Craig, L. C., and Pulley, A. O. (1962), *Biochemistry 1*, 89.

De Jager, E., Homan, J. D., and De Wied, D. (1963), *Can. J. Biochem. 41*, 1455.

Goldstein, J., and Craig, L. C. (1960), *J. Am. Chem. Soc. 82*, 1833.

Guidotti, G., Hill, R. J., and Konigsberg, W. (1962), *J. Biol. Chem. 237*, 2184.

Hausmann, W., and Craig, L. C. (1958), *J. Am. Chem. Soc. 80*, 2703.

Hill, R. J., and Konigsberg, W. (1960), *J. Biol. Chem. 235*, PC 21.

Hofmann, K., Yajima, H., Liu, T., and Yanaihara, N. (1962), *J. Am. Chem. Soc. 84*, 4475.

Kappeler, H., and Schwyzer, R. (1961), *Helv. Chim. Acta 44*, 1136.

King, T. P., and Craig, L. C. (1962), *Methods Biochem. Analy. 10*, 219.

Leach, S. J., and Scheraga, H. A. (1958), *Compt. Rend. Trav. Lab. Carlsberg 30*, 271.
Li, C. H. (1956), *Advan. Protein Chem. 11*, 101.
Li, C. H. (1962), *Recent Progr. Hormone Res. 18*, 1.
Li, C. H., Meinhofer, J., Schnabel, E., Chung, D., Lo, T., and Ramachandran, J. (1961), *J. Am. Chem. Soc. 83*, 4449.
Neumann, N. P., Moore, S., and Stein, W. H. (1962), *Biochemistry 1*, 68.
Rasmussen, H., and Craig, L. C. (1962), *Biochim. Biophys. Acta 56*, 332.
Rudman, D., Hirsch, R. L., Kendall, F. E., Seidman, F., and Brown, S. J. (1962), *Recent Progr. Hormone Res. 18*, 89.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Squire, P. G., and Li, C. H. (1961), *J. Am. Chem. Soc. 83*, 3521.
Sydnor, K. L., and Sayers, G. (1953), *Proc. Soc. Exptl. Biol. Med. 83*, 729.
Tsugita, A., and Akabori, S. (1959), *J. Biochem. 46*, 695.

A New Thioglucoside, (*R*)-2-Hydroxy-3-butenylglucosinolate from *Crambe abyssinica* Seed*

M. E. Daxenbichler, C. H. VanEtten, and I. A. Wolff

ABSTRACT: The major thioglucosidic constituent of seed from *Crambe abyssinica* Hochst ex R. E. Fries was isolated and shown to be a salt of (*R*)-2-hydroxy-3-butenylglucosinolate. This thioglucoside differs only in stereochemical configuration from the glucorapiferin (progoitrin) which occurs in a number of related plants in the mustard family. On enzymatic hydrolysis the new thioglucoside is converted to the enantiomer of goitrin, a physiologically active degradation product of glucorapiferin.

An example is thus provided of the occurrence in nature, in closely related species, of substances differing only in configuration at a single asymmetric center.

Seed from *Crambe abyssinica* Hochst ex R. E. Fries contains relatively large amounts of thioglucosides, including one or more that yields oxazolidinethione-type compounds on mustard myrosinase hydrolysis (VanEtten *et al.*, 1965). Thioglucoside hydrolysis to yield oxazolidinethiones has also been demonstrated to occur microbiologically (Greer, 1962). Since defatted *Crambe* seed meal may be used as a component of animal feeds and since oxazolidinethiones are known to possess physiological activity, it seemed desirable to investigate the thioglucosides of *Crambe*. Kjaer (1960) reviewed the occurrence of thioglucosides in the crucifer and closely related botanical families, and he showed the considerable diversity of their aglycons. We deal with the isolation and chemical characterization of the principal thioglucoside of *Crambe* seed.

Experimental

Crude Thioglucoside Preparation. Mature *Crambe* seed was obtained from a 1961 crop grown in Texas. The separation of thioglucosides from the crude extract of seed meal was similar to that employed by Kjaer *et al.* (1956). After removal of the pericarp the seed was flaked, the flakes were pentane-hexane extracted at room temperature, and the meal was ground to pass a 100-mesh screen. A 165-g sample of air-dried meal was shaken for 1 hour with 1500 ml of 80% (v/v) acetone-water. The solids were then removed in a continuous-type centrifuge. The residue meal was reextracted with two 750-ml volumes of the solvent. The combined aqueous acetone extracts were concentrated *in vacuo* at 45° to a volume of about 300 ml. After standing at refrigerator temperature overnight the precipitate that formed was removed by centrifugation. A 36 × 3.5-cm column was prepared from a water suspension of 300 g of acidic alumina (Camag) and washed with 1–2 liters of water. The supernatant extract was passed through the anionotropic column followed by a water wash of 2–3 liters. The thioglucosides were eluted with a 1% K_2SO_4 solution. Those eluate fractions (20-ml volumes) which contained thioglucosides were combined. The thioglucoside-containing fractions were

* From the Northern Regional Research Laboratory, Peoria, Ill. *Received September 28, 1964.* Presented at the Division of Agricultural and Food Chemistry, 147th meeting, American Chemical Society, Philadelphia, Pa., April 5–10, 1964. The Northern Regional Research Laboratory is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.