standstill after 20 minutes, when 5.1 ml. (1.05 mole equivalents) had been absorbed. The hydrogenation product was isolated with chloroform and the resulting crystalline product recrystallized from acetone–hexane. A total of 61 mg. of the 4,5-dihydro derivative was obtained which melted at 199–200°, $[\alpha]^{23}D$ +160° (*c* 0.65), $\lambda_{max}^{alc.}$ 288 mμ (95); reported[22] m.p. 195–197°, $[\alpha]D$ +165° ($CHCl_3$).

Anal. Calcd. for $C_{23}H_{32}O_6$ (404.49): C, 68.29; H, 7.97. Found: C, 68.08; H, 7.84.

Mesylation of the 4,5-Dihydro Derivative of XVIII and Preparation of the Triene XXIVa.—To a solution of the 4,5-dihydro derivative of XVIII (300 mg.) in pyridine (5 ml.) was added at 0° methanesulfonyl chloride (0.12 ml.) and the mixture allowed to remain at 0° for 16 hours. The resulting amorphous mesylate XVIIIc (390 mg.) was refluxed in pyridine (13 ml.) for one hour and the bulk of the pyridine removed *in vacuo*. The triene XXIVa was isolated with chloroform and the residual solid (345 mg.) crystallized from 95% alcohol. The crude triene (150 mg.) after additional crystallization from the same solvent melted at 183–184°, $[\alpha]^{23}D$ 126° (*c* 0.94), $\lambda_{max}^{alc.}$ 271 mμ (3,800); λ_{max}^{Nujol} 2.89, 5.71, 5.78, 5.84 and 5.89 μ; reported[22] m.p. 180–185°, $[\alpha]^{CHCl_3}D$ +79°.

Anal. Calcd. for $C_{23}H_{30}O_5$ (386.47): C, 71.48; H, 7.82. Found: C, 71.43; H, 7.92.

NEW BRUNSWICK, N. J.

[CONTRIBUTION FROM THE BIOCHEMICAL RESEARCH DEPARTMENT, THE ARMOUR LABORATORIES]

Studies on Adrenocorticotropin. XIV. Action of Bovine Fibrinolysin and of Liver Cathepsin on Corticotropin-A: Effect on Biological Activity

BY W. F. WHITE AND A. M. GROSS

RECEIVED AUGUST 6, 1956

Bovine fibrinolysin splits corticotropin-A after arginine in position 8 and after lysine in position 15 with complete loss of physiological activity. It appears reasonable to conclude that this is the mechanism of the destruction of ACTH by blood *in vitro*. Bovine liver cathepsin splits corticotropin-A after leucine in position 31, phenylalanine in position 35 without loss of physiological activity. Thus bovine liver cathepsin appears not to be responsible for the destruction of ACTH by extracts of liver. In its action on corticotropin-A, fibrinolysin resembles trypsin and liver cathepsin resembles pepsin, although in both cases the tissue enzyme has fewer points of attack than the gastro-intestinal enzyme.

Introduction

It has been shown that both blood[1] and extracts of liver[2] are capable of inactivating ACTH *in vitro*. In the case of both tissues, there is accessory evidence supporting the conjecture that inactivation might be due to proteolytic action.[3,4] The availability of purified fibrinolysin from bovine blood and of purified cathepsin from bovine liver has made it possible to study two new proteolytic agents for their effect on corticotropin-A. In addition to clarifying the relationship of the purified enzymes to the crude ACTH-destroying systems, this study has provided data concerning the specificity of the two enzymes.

Preparations. Corticotropin-A.—This material was prepared from porcine oxycellulose ACTH by chromatography on finely divided carboxylic-type ion-exchange resin using a slight modification of the process previously described.[5] By the use of a slightly lower *p*H (8.35 *vs.* 8.50), chromatograms resembling those of Dixon and Stack-Dunne[6] were obtained in which the major active peak was well separated from the other two active peaks and the unretarded inert peak. On countercurrent distribution of the major peak in the 2-butanol:0.2% aqueous trichloroacetic acid system,[7] little or no impurity was revealed and therefore the chromatographic fraction was used without further purification. The activity of such materials is 100–125 units per mg. when administered intravenously and 200–250 units per mg. when administered subcutaneously. All assays are done by the U. S. P. method.[8]

Fibrinolysin.—The preparation used was No. R_x 0345, obtained from Dr. Eugene C. Loomis of Parke Davis & Company, Detroit, Michigan. The proteolytic activity was approximately one casein unit per mg. nitrogen.[9]

Liver Cathepsin.—This enzyme was obtained from Dr. Kenneth C. Robbins of The Armour Laboratories and was prepared from bovine liver by means of a five-step procedure using the conventional alcohol precipitation technique.[10] The purification was followed by means of the proteolytic assay of Anson[11] and the final product has an activity of 0.39 mg. of liberated tyrosine per mg. dry weight. The activity against hemoglobin was not influenced by cysteine and the enzyme has no appreciable activity against the synthetic substrates for trypsin, chymotrypsin or pepsin.

Methods.—Small scale chromatography was done on Whatman #1 paper and large scale chromatographic separations were made on washed[12] Whatman #3 paper. Solvent systems used were the *n*-butanol:acetic acid:water (4:5:1) system of Partridge and the 2-butanol:aqueous ammonia (3:1) system.[13] Amino acid assays were done chromatographically by the technique of Roland and Gross.[14] Paper electrophoresis was done with the apparatus of Durrum.[15] N-Terminal determinations with dinitrofluorobenzene (DNFB) were carried out according to Sanger and Thompson[16] and the dinitrophenylated amino acids (DNP-amino acids) were identified by the two-dimensional chromatographic method of Levy.[17] The carboxypeptidase used for C-terminal work was a commercial 6 times crystallized product (Armour Laboratories) and the amino-peptidase used for N-terminal work was a purified intestinal prepara-

(1) G. Pincus, T. F. Hopkins and O. Hecter, *Arch. Biochem.*, **37**, 408 (1952).

(2) I. I. Geschwind and C. H. Li, *Endocrinol.*, **50**, 226 (1952).

(3) E. B. Astwood, M. S. Raben and R. W. Payne, *Recent Progr. in Hormone Research*, **VII**, 1 (1952).

(4) H. H. Tomizawa and R. H. Williams, *J. Biol. Chem.*, **217**, 685 (1955).

(5) W. F. White and W. L. Fierce, THIS JOURNAL, **75**, 245 (1953).

(6) H. B. Dixon and N. P. Stack-Dunne, *Biochem. J.*, **61**, 483 (1955).

(7) W. F. White, THIS JOURNAL, **75**, 503 (1953).

(8) "U. S. Pharmacopeia," Vol. XV, p. 176.

(9) L. F. Remmert and P. P. Cohen, *J. Biol. Chem.*, **181**, 431 (1949).

(10) K. C. Robbins, R. Schlueter and J. Shields, in manuscript.

(11) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

(12) Washed by irrigation with 0.1 *N* formic acid. The paper is then thoroughly air-dried before use.

(13) The 2-butanol:ammonia system is used in extended (48–60 hr.) runs with the solvent entering an absorbent pad at the bottom of the sheet. Thus true R_f values are not obtainable. The movement of the peptides is expressed in terms of the nearest amino acid in the standard mixture, which is always run at the sides of the sheet. *Cf.* ref. 14 for details on the use of this system.

(14) J. F. Roland and A. M. Gross, *Anal. Chem.*, **26**, 502 (1955).

(15) E. L. Durrum, *J. Colloid Sci.*, **6**, 274 (1951).

(16) F. Sanger and E. O. Thompson, *Biochem. J.*, **53**, 353 (1953).

(17) A. L. Levy, *Nature*, **175**, 126 (1954).

tion made by the method of Smith.[18] Both exopeptidases were treated with diisopropyl fluorophosphate (DFP) before use. Digests were chromatographed in the 2-butanol: ammonia system against amino acid standards. Wherever there was doubt concerning the identity of a spot with respect to the amino acid standards, supplemental portions of the digest were chromatographed and sections in the questionable locations were eluted, hydrolyzed with acid and rechromatographed. In this way it was possible to rule out interference from subtractive products of the exopeptidase action on the peptide fragments.

Experimental

Preliminary Observations on Digestion of Corticotropin-A by Fibrinolysin.—Small scale runs were made by taking up 0.05 μM samples of corticotropin-A in 0.1 ml. of 0.1 M ammonium acetate buffer containing 0.04 mg. of fibrinolysin. Digestion was done at 37° for 4, 8, 16 and 24 hr. at both *p*H 6.0 and 7.75. The digests were chromatographed as single spots on Whatman #1 paper in the 2-butanol: ammonia system, in which the intact corticotropin-A is immobile. No significant differences were noted between the two *p*H's. At 4 hr., in addition to the spot at the origin, a new spot appeared near the composite serine/glycine spot in the standards. At 8 hr., a second new spot appeared at proline. The two new spots appeared to attain maximum values by 16 hr., as shown by identical colorimetric readings[19] on the corresponding spots in the 16 and 24 hr. digests. No new spots appeared on longer digestion. On the basis of these data, it was decided to make large scale runs for 16 hr. at *p*H 7.75.

Large Scale Digestion of Corticotropin-A by Fibrinolysin.—A 10.0-mg. (net peptide) sample of corticotropin-A was taken up in 0.1 N ammonium acetate buffer at *p*H 7.75. As usual at this *p*H, not all of the corticotropin-A dissolved. Fibrinolysin in the amount of 2.33 mg. was added and the volume of the suspension was made to 4 ml. A few drops of toluene were added and the mixture was shaken gently at 37°. After a few hours the solution cleared up and the shaking was discontinued; however, the clear digest was allowed to remain at 37° for a total of 16 hr. A one-tenth aliquot of the digest was withdrawn for assay. Both the assay portion and the remaining bulk of the digest were immersed in boiling water for 5 min. to terminate enzyme activity. Both portions were then lyophilized.

Assay of Fibrinolytic Digest of Corticotropin-A.—The one-tenth aliquot (equivalent to 1.0 mg. of corticotropin-A) of the large scale digest referred to above was submitted for adrenocorticotropic activity by the U. S. Pharmacopeia method.[8] No depletion of adrenal ascorbic acid was found when the sample was tested at an assumed potency of 5 units. This result was interpreted as indicating that the 1.0-mg. sample could not have more than one unit of activity remaining. By contrast, another 1.0-mg. sample of corticotropin-A incubated in the *p*H 7.75 buffer *without* enzyme showed 170 units by the same assay. Thus at least 99% of the physiological activity was destroyed by the enzymatic action.

Separation and Identification of Fibrinolytic Fragments of Corticotropin-A.—Following the procedure used in this Laboratory for the separation of peptide fragments, the bulk of the digest was separated by means of two uni-dimensional chromatograms on Whatman #3 paper. The digest (equivalent to 9.0 mg. corticotropin-A) was first spread across an 8 in. strip of paper, leaving room only for a spot of the amino acid standard on each edge. The chromatogram was developed descending in the 2-butanol:ammonia system for 60 hr. After drying the sheet thoroughly in a stream of warm air, the standards and a narrow section of the digest were cut off the chromatogram and developed with ninhydrin. As in the preliminary runs, only three prominent ninhydrin-positive spots were visible in the digest: one spot opposite proline, a second near serine/glycine, and a third at the origin. Using the test strips as guides, the remainder of the 8 in. strip was cut and the segments were eluted with water. The eluates were concentrated *in vacuo* and each fraction was again spotted across a 5 in. strip of Whatman #3 paper. This time the chromatograms were developed in the Partridge system for 20 hr. By developing test strips it was found that each of the original spots gave only a single spot in the second system, although the one which was immobile in the first system spread out over a large area. The two narrow spots were cut and eluted as usual, while the large spot was divided into three roughly equal sections and each section was eluted separately. The chromatographic data for the fragments are given in Table I. Small samples of the first two spots and of the components of the diffuse third spot were re-run in the chromatographic system of Waley and Watson[20] and were also subjected to paper electrophoresis at *p*H 6.8 in 0.1 N ammonium acetate. The results, which are listed in columns 4 and 5 of Table I, tended to prove that fragments 1 and 2 were homogeneous and different from one another and that the separated portions of spots 3 were identical and therefore represented a single peptide fragment. This conclusion was further strengthened by the amino acid compositions as revealed by complete acid hydrolysis, as shown in column 6 of Table I. For this analysis, one-tenth aliquots of the fragments were taken up in 0.1 ml. of 6 N hydrochloric acid, sealed in a capillary tube and heated in an oven for 16 hr. at 105°. After drying *in vacuo* over phosphorus pentoxide and sodium hydroxide, the hydrolysates were chromatographed and assayed against amino acid standards.

The amino acid data permitted the conjecture that only two splits had occurred in the corticotropin-A molecule: (1) after arginine in position 8 and (2) after lysine in position 15. As can be seen in Fig. 1, these splits have already been shown to occur in corticotropin-A by the action of trypsin.[21] However, a third split made by trypsin at position 21 apparently did not occur with fibrinolysin. In order to clarify this conclusion, the three fragments of Table I were subjected to end-group analysis. The results, which are shown in the last column of Table I, were confirmatory.

In order to clarify the relationship between fibrinolytic fragment No. 3 and the fragments produced by tryptic action on intact corticotropin-A, a portion of fragment No. 3 was treated with trypsin.[22] The digest was chromatographed in the Waley and Watson system. Instead of the original spot at R_f = 0.37, two new spots appeared of which one had an R_f = 0.8 Lys, corresponding to that of fragment T_b of Fig. 1.[21]

Effect of Inhibitors on the Reaction between Fibrinolysin and Corticotropin-A.—Several inhibitors which had been studied by other investigators in the fibrinolytic system were tried for their effect on the reaction involving corticotropin-A. The action of the inhibitors was judged by the intensity of the ninhydrin spot opposite proline on chromatograms of the digest. The results are shown in Table II. In these experiments, the enzyme was made up at 0.4 mg. per ml. of 0.1 N ammonium acetate at *p*H 7.75. The amount of inhibitor shown in the second column of Table II was added to 0.1 ml. of the enzyme–buffer solution and the mixtures were incubated for 30 min. to ensure adequate contact between inhibitor and enzyme. The treated enzyme solutions were then added to 0.05 μM portions of corticotopin-A and incubated for 16 hr. The digests were dried and chromatographed on Whatman #1 paper in the 2-butanol:ammonia system.

Preliminary Observations on Digestion of Corticotropin-A by Liver Cathepsin.—Small-scale digests were made at *p*H 3.5 and at *p*H 7.5, using 0.1 N ammonium acetate buffers in each case. On chromatography of the digests in the 2-butanol:ammonia system, no mobile ninhydrin-positive spots were observed in the *p*H 7.5 digests, while in the *p*H 3.5 digests a new strong spot appeared at the reference position for arginine and a weak spot appeared slightly beyond proline. Serial tests at *p*H 3.5 at 2, 4, 6, 8 and 24 hr. indicated that both spots reached full intensity by the fourth hour and that no new spots appeared up to 24 hr. On the basis of these data it was decided to make a large scale run for 6 hr. at *p*H 3.5.

Large-scale Digestion of Corticotropin-A with Liver Cathepsin.—A 29.0-mg. sample of corticotropin-A was taken up in 5.0 ml. of 0.1 N ammonium acetate buffer at *p*H 3.5. At this *p*H, the corticotropin-A formed a clear solution. An aliquot was withdrawn for incubation without enzyme as an assay control. To the remainder of the solu-

(18) E. L. Smith, *J. Biol. Chem.*, **153**, 627 (1944).

(19) Colorimetric readings were made by means of a Densichron (W. M. Welch Mfg. Co., Chicago, Illinois).

(20) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

(21) W. F. White and W. A. Landmann, THIS JOURNAL, **77**, 1711 (1955).

(22) Armour Laboratories preparation No. 420-151.

TABLE I

DATA FOR PEPTIDE FRAGMENTS RESULTING FROM FIBRINOLYTIC ACTION ON CORTICOTROPIN-A

Spot (peptide) no.	*Rf* values in systems used for isolation 2-But.–NH_3	Partridge	*Rf* values in system of Waley and Watson	Movement in paper electrophoresis,[a] cm.	Amino acids on complete acid hydrolysis[b]	End group analysis
1	Ser/Gly[c]	0.40	0.47	+3.2	Ser(2), Tyr(1), Met(1), Glu(1), His(1), Phe(1), Arg(1)	DNFB: Ser
2	Pro	.22	.32	+5.3	Gly(2), Lys(2), Pro(1), Val(1)	Aminopeptidase: Try Carboxypep.....Gly.Lys
		0.25–0.30	.37	−1.5	Tyr(1), Gly(1), Lys(2), Arg(2).	
3	Zero	.30– .35	.37	−1.5	Pro(3), Val(2), Ala(3), Glu(3),	Aminopep.: Lys
		.35– .40	.36	−1.7	Asp(2), Leu(2), Phe(2)	Carboxypep.:...Leu.Glu.Phe

[a] Paper electrophoretic conditions: 16 hr. at 100 v. in 0.1 *N* ammonium acetate (*p*H 6.8). Endosmosis measured by dextran. As references, glutamic acid moved −8.2 cm. and lysine +8.5 cm. [b] The numbers in the parentheses refer to the relative numbers of residues of the amino acids as determined by photometric measurement of the ninhydrin spots. [c] *Cf.* ref. 13 (text).

TABLE II

EFFECT OF INHIBITORS ON THE REACTION BETWEEN FIBRINOLYSIN AND CORTICOTROPIN-A

Inhibitor	Amt. added to 0.1 ml. soln. containing 0.04 mg. fibrinolysin[a]	Rel. galv. reading on ninhydrin spot opposite proline
None		100
DFP (diisopropyl fluorophosphate)	0.5 μM	0
Pancreatic trypsin inhibitor (Kazal)[b]	4.0 mcg.	100
	40.0 mcg.	100
Soy bean trypsin inhibitor[b]	4.0 mcg.	0
Thioglycolate	0.2 μM	62
Thioglycolate	1.0 μM	25

[a] Buffer was 0.1 *N* ammonium acetate at *p*H 7.75. In each case the incubation mixture contained 0.05 μM corticotropin-A. [b] Both inhibitors prepared by Armour Laboratories.

TABLE III

DATA FOR PEPTIDE FRAGMENTS RESULTING FROM ACTION OF LIVER CATHEPSIN ON CORTICOTROPIN-A

Spot (peptide) no.	Paper electrophoresis: direction of movement at *p*H 7	*Rf* values in systems used for isolation 2-But.–NH_3	Partridge	Amino acids on complete acid hydrolysis[a]	End groups analy.
1	Anode	Arg[b]	0.63	Ala(2), Glu(1), Phe(1)	DNFB: Ala
2	Anode	Pro +	.82	Pro(1), Leu(1), Glu(1), Phe(1)	Isatin: Pro Carboxypep.: Phe
3	Cathode			Ser(2), Tyr(2), Met(1), His(1), Phe(1), Glu(3), Arg(3), Lys(4), Gly(3), Val(3), Pro(3), Asp(2), Ala(1), Leu(1)	

[a] The numbers in parentheses refer to the relative numbers of residues of the amino acids as determined by photometric measurement of the ninhydrin spots. [b] *Cf.* ref. 13 (text).

Fig. 1.—Sites of action of fibrinolysin and bovine liver cathepsin on corticotropin-A. For comparison, the previously determined sites of action of trypsin and pepsin are also shown. Solid arrows indicate a major site of action; broken arrows indicate secondary sites of action.

tion 1.5 mg. of bovine liver cathepsin was added as a solution in 0.5 ml. of the *p*H 3.5 buffer. After the addition of a small amount of toluene, both the control and the main solution were incubated at 37° for six hr. An aliquot was withdrawn for assay and then both portions were heated to terminate enzymatic action. The control and both digests were lyophilized.

Assay of Catheptic Digest of Corticotropin-A.—The cathepsin-treated sample and its control were assayed by the U. S. P. test. Within the limits of error of the assay, the two fractions showed identical activities.

Separation and Identification of Catheptic Fragments of Corticotropin-A.—In order to gain additional information regarding the electrophoretic behavior of the fragments of the digestion, a preliminary separation was made by paper electrophoresis in 0.1 *N* ammonium acetate (*p*H 6.8). The digest was divided into two parts and each half was spotted across a five inch strip of Whatman #3 paper. Between the two strips, a narrow reference strip was run with glutamic acid and lysine as controls. After 20 hr. at 100 v., the amino acid control strip and narrow cuts at the edges of the strips bearing the digest were developed with ninhydrin. Two spots appeared on the digest strips, one travelling toward the anode (3.9 cm. *vs.* 9.0 cm. for glutamic acid) and the other toward the cathode (3.6 cm. *vs.* 13.0 cm. for lysine). The spots were eluted, the corresponding ones for the two strips combined, and the eluates were dried. The acidic portion was resolved by successive unidimensional chromatograms in the two regular solvent systems. Only two significant spots emerged, corresponding to the two mobile spots observed in the preliminary runs. The chromatographic data are shown in the third and fourth columns of Table III. The basic portion was not chromatographed, but small portions were tested in the two solvent systems. No heterogeneity was revealed, although mobility was low in both systems. Semi-quantitative amino acid analyses were run on the fragments. The results are shown in Table III. The amino acid compositions plus the other data shown in the table were accepted as sufficient to characterize the first two fragments as identical with the two tetrapeptide fragments split off corticotropin-A by pepsin and constituting the last 8 amino acids at the C-terminus.[23] Since there was no evidence of the third peptide, Asp.Glu.Leu, previously shown[23] to be split off corticotropin-A by short-term peptic action, the third fragment of Table III appeared to constitute the remaining 31-positions of the amino acid sequence. To test this hypothesis, fragment no. 3 was subjected to the action of carboxypeptidase and of pepsin. In both cases, one-tenth aliquots of the fragment were subjected to one hour digestion with 25 μg. of the enzyme as solutions in 0.1 ml. of the appropriate buffer.[24] A chromatogram of the carboxypeptidase digest showed only leucine in the 2-butanol:ammonia system and the peptic digest showed only one new spot, which travelled at $R_f = 0.60$ in the Partridge system and slightly below glutamic acid in the 2-butanol:ammonia system. The latter data fitted the constants for the tripeptide, Asp.Glu.Leu, and the identity of this fragment was confirmed by isolation of a large quantity and application of the test used previously.[23]

Discussion

The finding that bovine fibrinolysin splits corticotropin-A after arginine in position 8 and after lysine in position 15 is in line with the observations of previous investigators,[25,26] who found that fibrinolysin attacks both arginine and lysine esters. The reason for the failure of fibrinolysin to split after lysine in position 21 (as does trypsin) is not clear, although it might be due to the low proteolytic activity of the fibrinolysin preparation. It is not possible to compare the relative rates of splitting by fibrinolysin and by trypsin for the three bonds of corticotropin-A since serial studies have not been made with trypsin at low levels of enzyme. Our studies with inhibitors support the conclusions of others[25,26] that fibrinolysin is similar to, but not identical with, trypsin in its proteolytic action. Both enzymes are inhibited by DFP, thioglycolate,[27] and soy bean inhibitor, but only trypsin is inhibited by the Kazal pancreatic inhibitor.[28]

There does not appear to be anything in the present work which would contradict the conclusion that fibrinolysin is involved in the destruction of ACTH by blood. The heat-labile ACTH-splitting component of blood has been traced to fraction III[29] which also contains fibrinolysin in the form of its precursor. Under normal conditions *in vivo*, fibrinolytic action is undoubtedly largely inhibited by antifibrinolysin. However, *in vitro* a slight disturbance of the balance, especially on addition of cofactor, could account for the observed destructive effect. Further tests of the blood system with the inhibitors studied in this work might be helpful in establishing the identity of fibrinolysin with the ACTH-destroying factor of Pincus, *et al.*[1]

The destruction of physiological activity by fibrinolysin was not surprising in view of previous studies. The activity of corticotropin-A appears to be associated with an intact N-terminal sequence,[30] but the unit comprising the first eight positions (which is present in both tryptic and fibrinolytic digests) is not sufficient.

Bovine liver cathepsin splits corticotropin-A at two of the five locations attacked by pepsin: after leucine in position 31 and after phenylalanine in position 35. Here again, the narrower specificity of the tissue enzyme might be due to the relatively low proteolytic activity of the preparation used.[31] This is especially true of the split after glutamic acid in position 5 and after glutamic acid in position 38, where pepsin splits only after long-term digestion with moderate concentrations of enzyme.[25,30,32] In its resemblance to pepsin, bovine liver cathepsin is similar to Proteinase I of lung.[33]

Retention of the physiological activity of corticotropin-A during the removal by cathepsin of a total of eight amino acid residues from the carboxyl end is in accord with the previous findings with pepsin. In the case of the latter enzyme, conditions have been found under which a total of 11 amino acid residues can be removed from the carboxyl end without loss of physiological activity.[23]

Since bovine liver cathepsin is without action on corticotropin-A at *p*H 7.5, and since it does not destroy activity even at its point of maximum proteolytic effect, it appears not to be responsible for the inactivation of ACTH by liver as observed by Geschwind and Li.[2] The failure of liver cathepsin

(23) W. F. White, THIS JOURNAL, **76**, 4194 (1954).

(24) The buffer for carboxypeptidase was *p*H 7.75, 0.1 *N* ammonium acetate; for pepsin, 0.1 *N* formic acid (*p*H 2.3).

(25) W. Troll, S. Sherry and J. Wachman, *J. Biol. Chem.*, **208**, 85 (1955).

(26) E. Rouwin and E. T. Mertz, *Federation Proc.*, **14**, 271 (1955).

(27) D. Grob, *J. Gen. Physiol.*, **29**, 219 (1946).

(28) N. H. Grant and K. C. Robbins, THIS JOURNAL, **77**, 2027 (1955).

(29) Personal communication from Dr. Gregory Pincus.

(30) W. F. White, THIS JOURNAL, **77**, 4691 (1955).

(31) Against hemoglobin, the liver cathepsin preparation was only about one-thirtieth as active as crystalline pepsin. Both enzymes were measured at their *p*H optima.

(32) W. F. White and W. A. Landmann, THIS JOURNAL, **77**, 771 (1955).

(33) A. M. Dannenberg, Jr., and E. L. Smith, *J. Biol. Chem.*, **215**, 55 (1955).

to attack either corticotropin-A or hemoglobin at *p*H 7.5 makes it unlikely that it is related to the liver enzyme of Tomizawa and Williams, which has been shown recently to have an effect on the corticotropin of sheep.[4]

CHICAGO 9, ILLINOIS

[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

In Vitro Conversion of Zymosterol and Dihydrozymosterol to Cholesterol

BY J. D. JOHNSTON AND K. BLOCH

RECEIVED OCTOBER 8, 1956

C^{14}-Zymosterol, prepared by incubation of yeast with labeled acetate, is shown to be converted to cholesterol by homogenates of rat liver. The process requires aerobic conditions and both the particulate fraction and the supernatant of liver homogenates. 24,25-Dihydrozymosterol can be transformed to cholesterol, but this reaction does not appear to occur normally in cholesterol biogenesis.

Introduction

In the biogenesis of the steroids, lanosterol is the first recognizable cyclic intermediate.[1] It is known to be metabolized further to cholesterol[2] by a process involving an oxidative removal of the three methyl substituents,[3] the saturation of the isooctenyl side chain and a shift[4] of the 8,9-double bond to the 5,6-position. It is not clear as yet in what order these transformations occur. One clue to the sequence of steps is provided by the structure of zymosterol ($\Delta^{8,24}$-cholestadienol) which may be regarded as a 4,4,14-trisnorlanostadienol. It is worth noting that zymosterol is a constituent of the lipids of yeast but has not so far been encountered in animal sources. However, the possibility that zymosterol is more widely distributed cannot be excluded since experience has shown that many of the intermediates in steroid biogenesis ordinarily occur in amounts too small to be detected by conventional methods. It therefore seemed worth investigating whether zymosterol, though hitherto considered to be a mycosterol, takes part in cholesterol biogenesis. For this purpose, we have employed the same procedures which have previously led to the recognition of squalene[5] and lanosterol[2] as cholesterol precursors. C^{14}-Zymosterol was prepared by biosynthesis and shown to yield cholesterol on incubation with liver homogenates. This transformation occurs also with the 24,25-dihydro derivative of zymosterol. The zymosterol–cholesterol conversion requires the particulate fraction as well as the soluble portion of liver homogenates for catalysis. In view of the fact that the net chemical changes involved are a reduction and the shift[4] of a double bond, it is of particular interest that aerobic conditions are essential for the over-all process.

Schwenk, *et al.*, have described the isolation of C^{14}-zymosterol[6] from yeast and have reported briefly[7] on the conversion of zymosterol to cholesterol in the whole animal.

(1) T. T. Tchen and K. Bloch, THIS JOURNAL, **78**, 1516 (1956).
(2) R. B. Clayton and K. Bloch, *J. Biol. Chem.*, **218**, 319 (1956).
(3) J. A. Olson and K. Bloch, *Federation Proc.*, **15**, 323 (1956).
(4) The term shift is used here to indicate the net change without reference to the mechanism involved.
(5) R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **200**, 135 (1953).
(6) E. Schwenk, G. J. Alexander, T. H. Stoudt and C. A. Fish, *Arch. Biochem. Biophys.*, **55**, 274 (1955).
(7) E. Schwenk, G. J. Alexander, C. A. Fish and T. H. Stoudt, *Federation Proc.*, **14**, 752 (1955).

Preparation of C^{14}-Zymosterol.—According to the observations of Klein,[8] the synthesis of sterols from acetate by baker's yeast is slow under anaerobic conditions. However, on exposure of anaerobically grown yeast to air or oxygen, non-saponifiable materials including ergosterol accumulate in considerable quantity. In the present experiments, anaerobically grown baker's yeast was briefly exposed to air, transferred to a nitrogen-free medium and incubated under oxygen. When the cells were harvested after three hours and extracted, squalene was the principal radioactive constituent of the lipid fraction. In contrast, after incubation for 34 hours, the yeast cells contained, apart from C^{14}-squalene, labeled zymosterol and ergosterol in considerable quantity (Fig. 1). The appropriate radioactive column fraction (III, Fig. 1), was

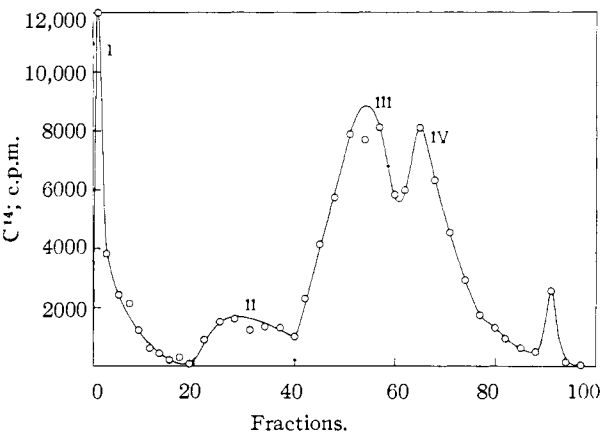

Fig. 1.—Chromatographic separation of non-saponifiable materials from yeast incubated with C^{14}-acetate: I, squalene; II, lanosterol; III, zymosterol; IV, ergosterol.

mixed with authentic zymosterol, crystallized and the specific activity of crystals and mother liquor material determined. These crystallizations were repeated with the acetyl and benzoyl derivatives. In all cases, the radioactivity remained associated with the crystalline carrier. Moreover, the elution diagram obtained by chromatography of the radioactive yeast sterol with carrier zymosterol showed satisfactory coincidence between radioactivity and the weight of solid material. These criteria furnish adequate proof for the identity of

(8) H. P. Klein, *J. Bacteriology*, **69**, 620 (1955).