[CONTRIBUTION FROM THE U. S. NAVAL MEDICAL RESEARCH INSTITUTE]

The Acetylcholinesterase Surface. VI. Further Studies with Cyclic Isomers as Inhibitors and Substrates[1,2]

BY S. L. FRIESS AND H. D. BALDRIDGE

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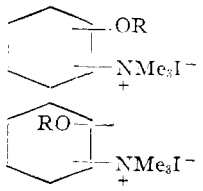
In further study of topographical features of the enzyme acetylcholinesterase from electric eel tissue, the *cis* and *trans* isomers of *d,l*-2-trimethylaminocyclopentanol iodide and the corresponding acetates have been prepared and tested for their activities as enzymatic inhibitors and substrates, respectively. The inhibition data indicate that the *cis*-alcohol offers a slightly better fit to the catalytic surface than its *trans* counterpart, with both derivatives possessing greater power than choline. Similarly, on the basis of two criteria of acceptability, the *cis*-acetate is a better substrate than the *trans*, and both surpass the natural substrate acetylcholine in fit to the catalytic surface. The body of evidence from both the cyclopentane and cyclohexane derivatives is in accord with a maximum distance of about 2.5 Å. between anionic and esteratic sites on the surface.

Some preliminary work[3] directed toward estimation of the separation distance between the anionic and esteratic sites of electric eel cholinesterase (AChE) made use of the *cis* and *trans* isomeric pairs I–III and II–IV as inhibitors and substrates, respectively, of the enzyme. However, the N–O in-

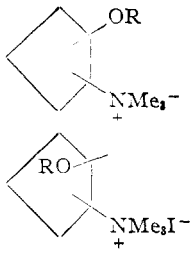


R = H, R = $COCH_3$

I II

III IV

teratomic distances[4] in these cyclic derivatives of choline and its acetate could only be estimated to within a 0.4–0.8 Å. range, corresponding to extremes of models in which both chair and boat forms of the six-ring and equatorial and polar disposition of its substituents were considered. Now, in an attempt to reduce this uncertainty range in spatial separation of the polar functions of these inhibitors and substrates, and also produce further controlled variations in this distance for more precise probing of the inter-site distance on the enzyme, the corresponding alcohols (V, VII) and acetates (VI, VIII) in the cyclopentane series have been prepared and tested for their enzymatic potency. In these deriv-



R = H R = $COCH_3$

V VI

VII VIII

atives the virtual planarity[5] of the five-ring was taken as leading to unique disposition of its substituents, and interatomic N–O distances were calculated assuming tetrahedral distribution of these substituents at the ring carbons. As previously,[3] the relative degree of acceptance of inhibitor or substrate by the enzyme is interpreted as an index of the nearness of matching of the N–O distance in the small molecule to the average anionic-esteratic separation distance on the protein surface, with the implicit assumption that binding of the polar substituents to the polar protein surface is not appreciably perturbed (over the binding of acetyl choline or choline as basic structures) by the presence of the three extra ring methylene groups.

Results

Inhibitors.—Inhibition of the enzymatically catalyzed hydrolysis of acetylcholine chloride (AC) by the cyclic alcohols V and VII was found to be comparable in strength to that exhibited by the six-membered alcohols I and III, or choline itself, but certain graded differences in power are to be noted. Values for the enzyme–inhibitor dissociation constants K_I (derived from the P. W. Wilson inhibition plots[6] of v/v_I *vs.* inhibitor concentration) at 25.14°, which can be taken as a measure of the relative effectiveness of inhibition are given for compounds V and VII in Table I below, together with the calculated N–O separation distances in the individual quaternary aminoalcohols. Also included in the table for reference are the data previously obtained for the six-membered cycles I and III.

TABLE I

ENZYME–INHIBITOR DISSOCIATION CONSTANTS

25.14 ±0.03°, *p*H 7.3, μ = 0.140

Compound	Ring size	$K_I \times 10^4$	O–N distance,[a] Å.
I (*d,l-cis*)[b]	6	1.1 ± 0.1	2.5–2.9[c]
III (*d,l-trans*)[b]	6	2.1 ± .1	2.9–3.7
V (*d,l-cis*)	5	0.75 ± .05	2.51
VII (*d,l-trans*)	5	0.89 ± .06	3.45

[a] Calculated on the basis of tetrahedral angles for substituents at each ring carbon and using the following single bond distances: C–C, 1.54 Å.; C–O, 1.43 Å.; C–N, 1.47 Å. [b] See reference 3. [c] See reference 4.

It is seen that in each isomeric pair, with decreasing values of K_I denoting increasing inhibitory power, the *cis* derivative is a slightly more potent inhibitor of the enzyme than its *trans* isomer. In each pair too the calculated N–O distance is smaller for the *cis* compound than for the *trans*. Moreover,

(1) The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department.

(2) Presented in part at the Third International Congress of Biochemistry, Brussels, Belgium, Aug. 1–6, 1955.

(3) H. D. Baldridge, W. J. McCarville and S. L. Friess, THIS JOURNAL, **77**, 739 (1955).

(4) Given by G. E. McCasland and D. A. Smith, *ibid.*, **72**, 2190 (1950).

(5) J. E. Kilpatrick, K. S. Pitzer and R. Spitzer, *ibid.*, **69**, 2483 (1947).

(6) P. W. Wilson, "Respiratory Enzymes," H. A. Lardy, Ed., Burgess Publishing Co., Minneapolis, Minn., 1949, p. 23.

within the restricted range of variation in K_I produced by the relatively small structural changes in this series, compound V emerges as about the best inhibitor of the group with the additional property of possessing the smallest N–O separation distance.

However, one curious anomaly to be noted in Table I is that compound VII deviates from the regular correlation of increasing inhibitory power with decreasing O–N separation distance exhibited in the series I–III–V, with the positions of I and VII showing apparent inversion through small differences in their K_I values. To some extent, this may reflect small differential contributions of the two carbocyclic rings to over-all binding with the protein surface.

Consequently, these observations do not uniquely lead to a value for an optimum N–O separation distance, assuming[7] that both polar functions are involved in binding to the two sites of a catalytic unit on the enzyme's surface. However, a limiting distance of about 2.5 Å. for this separation distance would be in accord with the correlation in the inhibitor series I–III–V and also with the observation that choline itself (with a K_I value[8] of 4.5×10^{-4} at 25°, as verified under the present experimental conditions) is a weaker inhibitor than any of the series I, III, V, VII and has an approximate functional separation distance[3,9] of about 2.3 Å. based on the Eyring formulation for a flexible chain.

Substrates.—The enzymatic hydrolysis of acetates VI and VIII was studied under conditions of fixed *p*H and enzyme concentration identical to those used[3] in work on II and IV. In particular, the nature of each initial substrate concentration *vs.* activity profile was determined in the region of its curve maximum, leading to information on the position and the height of the maximum. Height data in each case were then compared with the peak value for acetylcholine as the standard substrate. Figure 1 presents the concentration–activity curves for VI and VIII at 25.12° and *p*H 7.3. The essential data on position of the substrate maxima and the relative rates at these optimum points are summarized in Table II, which also includes for comparison the corresponding data previously tabulated for compounds II and IV.

Some interesting points arise from inspection of these data. First, on comparing the two pairs of acetate isomers, a regular relation is seen to exist just as in the corresponding inhibitor alcohols; in each pair the *cis* ester reaches its optimum rate at a slightly lower concentration than does its *trans* isomer, and at their respective optima each *cis* isomer

(7) This assumption is open to question on the grounds that these quaternary aminoalcohols display only the same order of inhibitory strength as the monofunctional tetraalkylammonium ions.[8] However, if only the quaternary amino function in compounds I–III–IV–VII were involved in the reversible binding, it would be difficult to (1) rationalize any change in K_I on proceeding from one member of this series to another, (2) explain the relative order (*cis* > *trans*) of power within isomeric pairs, and (3) account for the striking resemblance between the *cis-trans* correlation for the cyclic inhibitors and that for the cyclic substrates.

(8) I. B. Wilson, *J. Biol. Chem.*, **197**, 215 (1952).

(9) For further indication that the acetylcholine molecule (and hence choline) is not long enough to be a good complementary model of the catalytic surface unit, see S. A. Bernhard, THIS JOURNAL, **77**, 1966 (1955).

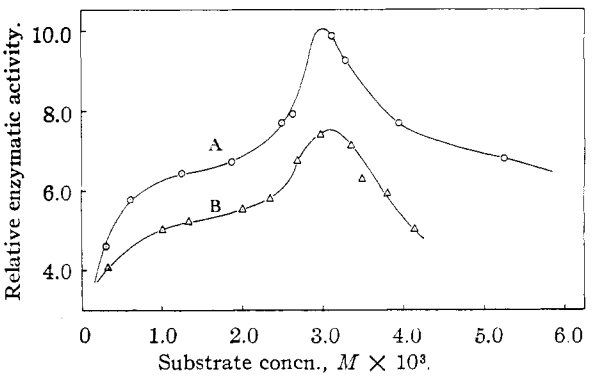


Fig. 1.—Substrate activity *vs.* initial concentration curves: A, compound VI; B, compound VIII.

evokes higher hydrolytic activity from the enzyme than the *trans* compound. If one takes these previously discussed[3] criteria (lowness of concentration to achieve maxima, relative activity at the maxima) as indicative of the degree of acceptance of substrates by the catalytic surface, then the same general conclusions may be drawn from these stereospecific substrates as were inferred above from the inhibitors, namely, that (1) each *cis* derivative represents a better fit to the surface than its *trans* isomer, and (2) the entire series of cyclic esters function as more active substrates for this enzyme than the natural substrate AC, just as the cyclic alcohols are better inhibitors than choline. The implication again is that the *cis* isomers with their shorter N–O separation distance afford a better complementary fit to the surface than the *trans* compounds. In support of this view, the *cis*-cyclopentane derivative VI with the shortest calculated N–O distance of 2.5 Å. (see Table I for N–O distances applicable to alcohols or acetates) has markedly the highest catalyzed hydrolysis rate, just as its parent alcohol V is the most powerful of the inhibitors of its class. However, the peak concentration criterion does not appear to hold outside of a given isomeric pair of substrates since, for example, the cyclopentane derivative VI characterized by such high activity has its maximum at a higher substrate concentration than does either of the isomers in the cyclohexane series.

TABLE II

QUATERNARY AMINOACETATES AS AChE SUBSTRATES

25.12°, *p*H 7.30

Substrate	Ring size	[Substrate]$_0$ at optimum, $M \times 10^3$	Relative activity at optimum
Acetylcholine		3.33	1.00
II (*d,l-cis*)[a]	6	1.75	1.14
IV (*d,l-trans*)[a]	6	2.14	1.06
VI (*d,l-cis*)	5	2.95	1.43
VIII (*d,l-trans*)	5	3.07	1.07

[a] See reference 3.

In part, the position of a given substrate maximum in these derivatives is related to a curious phenomenon illustrated in Fig. 1. In addition to the observed inhibition by excess substrate characteristic of choline-like esters and this enzyme, each of these curves has an apparent inflection

point on the low concentration side of the maximum. This appears in a branch of the curve with low slope values just preceding the take-off to the maximum, and the length of this branch (and hence the position of the maximum) varies from compound to compound. The concave-upward portion of this branch was also observed in the data for the lowest concentration points given previously[3] for compounds II and IV, but no such branch occurs in the published data[10] for acetylcholine hydrolysis. With the present preparations, a slight concavity in the acetylcholine concentration *vs.* activity plot was also observed on the low concentration side, which was obliterated in the customary log *c vs.* activity plot over a wide concentration range. No proven explanation is offered for this particular behavior at present, but the general shape of the curves appears consistent with at least one speculation that two or more very similar enzymes may be present in these preparations, one of which would tend to cause the curve to level out near the region of the inflection point (followed by a drop as inhibition by substrate sets in at higher concentrations) and the other of which leads to the second abrupt increase in height at its own characteristic substrate levels, followed by substrate inhibition. A summation of two such actions with appropriate proportions of these enzymes might lead to the observed curve shapes.

Experimental[11]

Synthesis of Substrates and Inhibitors

2-Chlorocyclopentanone.—Chlorine gas was passed through a rapidly stirred mixture of 150 g. of cyclopentanone and 400 ml. of water for 1.5 hr. at a bath temperature of 45–50°. The chloroketone product was extracted with ether and the ether layer then washed with saturated ammonium sulfate solution, dried over anhydrous sodium sulfate and rapidly distilled to give crude 2-chlorocyclopentanone. Fractionation of this material gave 39.8 g. (18%) of pure chloroketone, $n^{25}D$ 1.4790, b.p. 87° (19 mm.).

2-Dimethylaminocyclopentanone.—A solution of 20 g. of 2-chlorocyclopentanone in 50 ml. of 95% ethanol was added with swirling to a cold solution of 50 g. of 33% aqueous dimethylamine in 50 ml. of 95% ethanol at such a rate that the temperature remained at 0–10°. The cold solution was placed in a refrigerator at about 10° for 48 hr. It was then acidified with hydrochloric acid, saturated with sodium chloride and washed with ether. The mixture was made basic with potassium hydroxide and the free aminoketone extracted with ether and dried over anhydrous magnesium sulfate. The filtered ether solution was concentrated *in vacuo* under nitrogen at about 50° (water-bath) and the crude yield (6.8 g.) fractionated to give 4.6 g. (19%) of pure 2-dimethylaminocyclopentanone, b.p. 84–86° (24 mm.), $n^{25}D$ 1.4592, picrate, m.p. 144–146° dec. The product proved to be quite unstable on standing, making it necessary to carry out reduction steps quite soon after its preparation.

Catalytic Reduction of 2-Dimethylaminocyclopentanone.—A solution of 3.27 g. of 2-dimethylaminocyclopentanone in 100 ml. of absolute alcohol was hydrogenated at room temperature over 0.5 g. of Adams catalyst at an initial pressure of 35 pounds per square inch. After four hours the catalyst was removed by filtration and the solution concentrated *in vacuo* at about 50° under nitrogen. Fractionation of the residue gave 0.42 g. (13%) of *cis*-2-dimethylaminocyclopentanol, b.p. 58–60° (11 mm.), $n^{25}D$ 1.4559, picrate m.p. 93–94°, methiodide[12] m.p. 146–147°, and 1.94 g. (59%) of *trans*-2-dimethylaminocyclopentanol, b.p. 94–95° (11 mm.), $n^{25}D$ 1.4710, picrate m.p. 172–173°, methiodide m.p. 206–207°.

Sodium Borohydride Reduction of 2-Dimethylaminocyclopentanone.—A solution of 1 g. of sodium borohydride in 10 ml. of water was added over a period of about 30 minutes to a stirred solution of 1.70 g. of the aminoketone in 15 ml. of water. Stirring was continued for an additional 30 minutes. The reaction mixture was made strongly basic with potassium hydroxide solution, saturated with potassium chloride and extracted with ether. The combined ether extract was dried over anhydrous magnesium sulfate, filtered and concentrated at reduced pressure under nitrogen. Fractionation of the crude yield gave 0.61 g. (35%) of *cis*-2-dimethylaminocyclopentanol, b.p. 60–61° (12 mm.), $n^{25}D$ 1.4552, which solidified on standing in the cold, m.p. 24–26°, and 0.55 g. (32%) of *trans*-2-dimethylaminocyclopentanol, b.p. 96–98° (12 mm.), $n^{25}D$ 1.4698.

Preparation and Reduction of 2-Dimethylaminocyclopentanone without Intermediate Isolation.—To a cooled (15–20° water-bath) solution of 47 g. of 33% aqueous dimethylamine in 50 ml. of 95% ethanol, swept by a stream of nitrogen gas, there was added with mechanical stirring over a period of 10 minutes a solution of 17.8 g. of 2-chlorocyclopentanone in 50 ml. of 95% ethanol. Stirring was continued for a period of about five hours, following which the solution was left at room temperature overnight. A solution of 3.75 g. of sodium borohydride in about 50 ml. of water was then added to the rapidly stirred reaction mixture at room temperature over a period of about 30 minutes, after which stirring was continued for two hours. The crude aminoalcohol product was extracted as described above and fractionated to yield 3.6 g. (18%) of *cis*-2-dimethylaminocyclopentanol, $n^{25}D$ 1.4550, and 1.53 g. (8%) of *trans*-2-dimethylaminocyclopentanol, $n^{25}D$ 1.4700.

***cis*-2-Dimethylaminocyclopentanol Methiodide (V).**—A mixture of 1.35 g. of *cis*-2-dimethylaminocyclopentanol and 10 g. of methyl iodide in about 100 ml. of absolute ether was placed on a mechanical shaker at room temperature. Precipitation of the methiodide was very slow; only cloudiness appeared after several hours, and the supernatant remained cloudy even after shaking for 20 hours. The crude methiodide was recrystallized twice from ether–methanol mixture to give 1.7 g. (59%) of *cis*-2-dimethylaminocyclopentanol methiodide, m.p. 146–147° after drying *in vacuo* at 100°, mixed m.p. with authentic[12] *cis*-methiodide (prepared as below) 145.5–146.5°.

Anal. Calcd. for $C_8H_{18}NOI$: C, 35.44; H, 6.69; N, 5.17; I, 46.81. Found: C, 35.55; H, 6.62; N, 4.97; I, 46.60.

***cis*-2-Dimethylaminocyclopentyl Acetate.**—Ketene was passed through a solution of 2.5 g. of *cis*-2-dimethylaminocyclopentanol in about 100 ml. of ether for two hours. The resulting mixture was filtered and fractionated to give 2.4 g. (73%) of *cis*-2-dimethylaminocyclopentyl acetate, b.p. 83–85° (13 mm.), $n^{25}D$ 1.4498.

***cis*-2-Dimethylaminocyclopentyl Acetate Methiodide (VI).**—A mixture of 2.4 g. of *cis*-2-dimethylaminocyclopentyl acetate and 10 g. of methyl iodide in 100 ml. of ether was mechanically shaken overnight. The crude precipitated salt was twice recrystallized from ether–methanol mixture and dried at 100° *in vacuo* to give 3.1 g. (71%) of *cis*-2-dimethylaminocyclopentyl acetate methiodide, m.p. 176–177°.

Anal. Calcd. for $C_{10}H_{20}NO_2I$: C, 38.35; H, 6.44; I, 40.52. Found: C, 38.20; H, 6.48; I, 40.71.

Authentic *cis*-2-Dimethylaminocyclopentanol Methiodide.—To a solution of 0.16 g. of *cis*-2-aminocyclopentanol hydrochloride (supplied by G. E. McCasland)[12] in about 25 ml. of absolute methanol, under reflux, three successive portions of 5 g. of methyl iodide and 5 g. of anhydrous potassium carbonate were added over a period of about 24 hours. Filtration and concentration of the filtrate from this mixture gave a residue which was repeatedly digested with hot chloroform. Concentration of the combined chloroform extract gave the crude methiodide which was recrystallized from

(10) See, for example, K. B. Augustinsson, *Arch. Biochem.*, **23**, 111 (1949); K. B. Augustinsson and D. Nachmansohn, *J. Biol. Chem.*, **179**, 543 (1949).

(11) Melting points and boiling points are uncorrected. Analyses by courtesy of Dr. W. C. Alford, Microanalytical Laboratory, National Institutes of Health, Bethesda, Md.

(12) Identical with the methiodide derived from an authentic sample of *cis*-2-dimethylaminocyclopentanol which was prepared *via* the oxazoline route and generously furnished by Dr. G. E. McCasland. See G. E. McCasland and D. H. Smith, THIS JOURNAL, **72**, 2190 (1950).

ether–methanol mixture and dried *in vacuo* at 100° to give authentic *cis*-2-dimethylaminocyclopentanol methiodide, m.p. 146–147°.

trans-**2-Dimethylaminocyclopentanol Methiodide (VII).**—A solution of 14 g. of purified cyclopentene oxide in 40 ml. of ethanol was mixed with a threefold excess of 33% aqueous dimethylamine, sealed into a glass bomb and heated at 120–130° (oil-bath) for 24 hours. After cooling, the bomb's contents were transferred to a separatory funnel with the aid of about 100 ml. of water, saturated with salt and extracted with ether. The ether extract was dried over anhydrous magnesium sulfate and fractionated to yield 12.7 g. of *trans*-2-dimethylaminocyclopentanol, b.p. 106° (16 mm.). Three grams of this aminoalcohol was then added to a solution of 13 g. of methyl iodide in 100 ml. of absolute ether and the resulting mixture shaken overnight. Precipitation of the methiodide was quite rapid, and a threefold recrystallization from ether–methanol mixture gave the pure product, m.p. 207–208°.

Anal. Calcd. for $C_8H_{18}NOI$: C, 35.44; H, 6.69; N, 5.17. Found: C, 35.14; H, 6.76; N, 4.96.

trans-**2-Dimethylaminocyclopentyl Acetate Methiodide (VIII).**—A solution of 5 g. of *trans*-2-dimethylaminocyclopentanol in 75 ml. of anhydrous ether was continuously stirred and bubbled with ketene for a period of four hours. The ethereal solution was then filtered, mixed with 20 g. of methyl iodide and shaken mechanically overnight. The precipitated methiodide was recrystallized repeatedly from methanol–ether mixture; m.p. 166–167°.

Anal. Calcd. for $C_{10}H_{20}NO_2I$: C, 38.35; H, 6.44; N, 4.47. Found: C, 38.48; H, 6.31; N, 4.61.

Enzymatic Rate Determinations.—The equipment and techniques employed were essentially those of a previous study.[3] A standard phosphate buffer of *p*H 7.3 and containing 0.01 *M* Mg(II) ion was used in both inhibitor and substrate work, with acetylcholine chloride serving as substrate in evaluation of inhibitor strength. The titration cell was thermostated at 25.12 ± 0.03°. The stock enzyme solution[13] from which aliquots were drawn and diluted for kinetic experiments assayed at 2.7×10^5 μmole acetylcholine hydrolyzed/hr./mg. protein, with initial substrate concentrations in the range $1–10 \times 10^{-3}$ *M* being employed.

Substrates and inhibitors were freshly recrystallized samples, with solutions being prepared and refrigerated just before use. Rate runs were restricted to points within the first 5–7% of reaction to avoid complications due to build-up of the weakly inhibitory alcohol products and corrected for non-enzymatically catalyzed hydrolysis of substrate. Rates were in general reproducible to ±5%. In the plots of v/v_I *vs.* inhibitor concentration to obtain K_I values, the maximum uncertainties in K_I noted in Table I were estimated from each respective least-squares fit of the data.

Acknowledgments.—We are indebted to Dr. H. S. Polin for his generous aid in procurement of electric eel tissue and its initial extracts and to Mr. J. Hockstad for valuable technical assistance.

(13) Prepared from electric eel tissue extracts according to the purification technique of D. Nachmansohn and M. A. Rothenberg, *J. Biol. Chem.*, **168**, 223 (1947).

BETHESDA, MD.

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

The Reaction between Concanavalin-A and Glycogen[1]

BY J. A. CIFONELLI, R. MONTGOMERY AND F. SMITH

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Glycogens from various biological sources give a precipitin reaction with concanavalin-A, a globulin from Jack Bean meal. It is found that certain glycogens can be differentiated from others by their capacity to react with concanavalin-A and that native starches including waxy maize as well as the A- and B-fractions of various starches differ from glycogens by their failure to give the reaction. Since removal of the outer branches of glycogen by β-amylase results in an increase in the precipitin reaction, there is reason to believe that the reaction involves primarily the inner portions of the molecules. The fact that all amylaceous polysaccharides do not precipitate concanavalin-A equally well thus suggests that they may not have identical inner structures.

Basic similarities among glycogens from different sources have been established by the use of various chemical and enzymatic procedures. Thus, methylation[2–5] of glycogens has indicated average unit chains of twelve α-D-glucose units linked through positions 1 and 4 and joined to each other through position 1 of the "reducing residue" to position 6 of a glucose residue in another chain. There is also evidence concerning the presence of other linkages.[6–9]

Periodate oxidation techniques[7,10,12–14] confirmed the presence of one terminal unit for approximately 12 D-glucose residues, though it has been claimed that certain glycogen samples may contain one terminal unit per chain length of six residues[14,15] while in other instances the number of end groups per chain has been reported as being as low as one in 18[10,11,14,16] or even one in 23 residues.[16]

An enzymatic method[17–19] has been employed to determine the ratio of branch points to end-groups and the position of the branch point in each unit chain. The results from this method were said to indicate that a regular series of glycogens exists with chain lengths varying from 10 or less units to approximately 18 D-glucose units.

Glycogen has been shown to give a precipitate when treated with concanavalin-A,[20] a globulin extracted from Jack Bean meal. The work herein

(1) Paper No. 3297, Scientific Journal Series, Minnesota Agricultural Experiment Station. This work constitutes part of a thesis submitted by J. A. Cifonelli to the Graduate Faculty of the University of Minnesota in partial fulfillment for the degree of Ph.D., 1953.

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(11) W. N. Haworth, E. L. Hirst and F. A. Isherwood, *ibid.*, 577 (1937).

(12) M. Abdel-Akher and F. Smith, THIS JOURNAL, **73**, 994 (1951).

(13) A. L. Potter and W. Z. Hassid, *ibid.*, **70**, 3488 (1948).

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(20) J. B. Sumner and S. F. Howell, *ibid.*, **115**, 583 (1936).