

Table 1. Ratios and amounts of total stable iodine and total stable thyroxine iodine in the thyroid glands of burros at death (mg/g). Mean \pm standard error.

	Group Ia	Group Ib	Control
Survival (days)	49.5 \pm 1.23	79.0 \pm 4.66	
Total iodine (TI)	6.4 \pm 2.8	1.20 \pm 0.22	5.56 \pm 1.98
Thyroxine (TX) iodine	0.70 \pm 0.27	0.24 \pm .44	0.66 \pm 0.22
TX/TI	.127 \pm .021	.212 \pm .044	.133 \pm .028

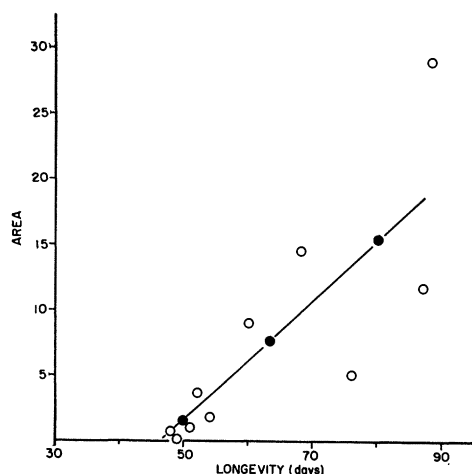


Fig. 2 Planometrically determined areas beneath the curves in Fig. 1a and b, expressed in arbitrary units, as a function of survival time. The black dots and connecting line represent the regression of area on longevity.

fell to subnormal levels (Fig. 1a). Those that accumulated iodine-131 survived significantly longer than did those within the normal range or below.

This relationship was not observed in the stable iodine values of thyroids taken at autopsy. The total stable iodine and the stable thyroxine iodine was least in those surviving the longest. In those surviving the shortest period of time both the stable iodine content and thyroxine iodine content were not significantly different from the normal controls (Table 1).

A significant correlation was apparent between the planometrically determined areas under the accumulation curves (Fig. 1a and b) and the survival time of the animals (Fig. 2).

The causal relationship between iodine-131 uptake and survival is not understood. It may be a reflection of degree of stress response to ionizing radiation. The early deaths may indicate a body-wide metabolic collapse, whereas later deaths indicate less irreparable damage and partial compensation for injury but eventual deaths from more latent physiologic disturbances.

References and Notes

- * Veterinary Corps, U.S. Army.
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6. The I^{131} was obtained from the Oak Ridge National Laboratory on allocation from the U.S. Atomic Energy Commission.
7. The stable iodine determinations were done by the Chaney Chemical Laboratories, Glendale, Calif.
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Demonstration of an Oligosaccharide Intermediate in the Enzymatic Hydrolysis of Cellulose

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Data summarized by Reese *et al.* (1), and Tracey (2) indicate a random cleavage of the cellulose molecule by cellulase. Such a random cleavage should yield a variety of intermediate dextrans with the eventual formation of cellobiose and glucose. However, the only products that have been found, in spite of concerted efforts, are cellobiose and glucose (3). This failure, plus the fact that cellobiose, but not glucose, inhibits the enzymatic hydrolysis of cellulose (1), has led Levinson *et al.* (4) to conclude that cellobiose is the end-product of cellulase action, in analogy to β -amylase, with glucose arising as the result of a β -glucosidase. This communication is to report the demonstration and tentative identification of an oligosaccharide intermediate in the enzymatic hydrolysis of cellulose.

Successful demonstration of the intermediate has been achieved repeatedly by enhanced dissociation of the oligosaccharide-enzyme complex through dialysis. After incubating cellulase and substrate in a collodion sac, an oligosaccharide, tentatively identified as cello-tetraose, was demonstrated in the dialyzate.

The enzyme used in these experiments was obtained by growing *Myrothecium verrucaria* (USDA 1334.2) on the medium of Saunders *et al.* (5) for 11 days at room temperature. Two liters of the culture filtrate was concentrated in vacuum to 50 ml and exhaustively dialyzed in a collodion sac against running tap and distilled water. No further purification was attempted. The cellulose substrate (Solka-floc) was swollen in phosphoric acid by the method of Walseth (6) and also dialyzed against tap and distilled water.

Two grams of substrate, suspended in 45 ml of distilled water, was placed in a freshly prepared collodion sac with 2 mg of dialyzed Difco proteose-peptone (7) and 5 ml of the concentrated enzyme solution. The collodion sac was suspended in 2 lit of 0.001M acetate buffer, pH 5.0. The external and internal solutions were stirred continuously at 50°C for 5 hr.

The dialyzate was concentrated to 10 ml in vacuum, and chromatographed on Whatman No. 1 filter paper using an isopropyl alcohol, water, and glacial acetic acid solvent (4). The chromatograms when developed with a benzidine spray (8) indicated large concentrations of glucose and cellobiose and the presence of another reducing sugar with a lower R_F . Five milliliters of the concentrate was strip-chromatographed in the afore-mentioned solvent, and reference strips were developed with the benzidine spray. The sections of the unsprayed chromatogram that the reference strips indicated to contain the higher saccharide were cut out and eluted with water. The eluate was concentrated in vacuum to 5 ml and rechromatographed. These chromatograms indicated the presence of a single reducing sugar. Control experiments of cellulose or enzyme alone failed to give rise to the observed higher saccharide. R_F values are given in Table 1.

Total sugars were determined as glucose by the anthrone method of Dimler *et al.* (9) and reducing sugars by the methods of Somogyi (10) and Nelson (11). The molar concentration of the saccharide was determined by comparing its reducing power with cellobiose rather than with glucose (12). From the molar concentration the molecular weight was calculated to be 635 (including corrections for the addition of water and filter paper blanks) as compared with a theoretical 666 for cellotetraose. This error of less than 5 percent is considered to be within the limits of the methods used. Further evidence that the new compound is the tetraose is the fact that when the R_M values (13) are plotted as a function of the number of glucose units per molecule, a straight line results (Fig. 1).

Evidence that the oligosaccharide is an intermediate in the hydrolysis of cellulose was obtained by incubating 1.0 ml of sugar solution containing 448 μ g with 0.1 ml of the concentrated enzyme at 50°C. Samples were withdrawn periodically and chromatographed in solvent B with the results shown in Fig. 2.

Since only very limited quantities (about 2 mg) of

Table 1. R_F values of the observed hydrolytic products of cellulose.

Sugar	Solvents*		
	A	B	C
Glucose	0.47	0.54	0.74
Cellobiose	.29	.41	.66
Higher saccharide	.08	.19	.45

* The solvents consisted of glacial acetic acid, isopropyl alcohol and water in the following ratios (V/V/V): A (10:67.5:22.5); B (10:58.5:31.5); C (10:49.5:40.5).

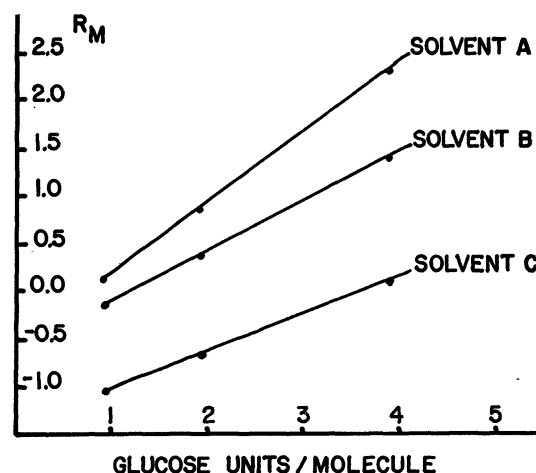


Fig. 1. Relationship of R_M values, $\ln(\frac{1}{R_F} - 1)$ and molecular size of the observed hydrolytic products of cellulose.

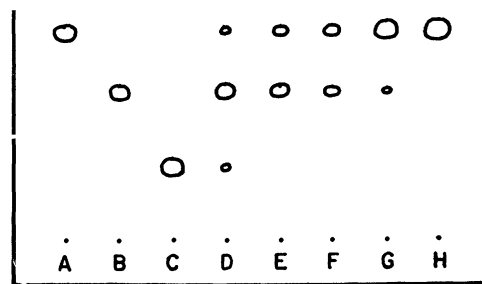


Fig. 2. Chromatographic analysis of oligosaccharide hydrolysis: A, known glucose; B, known cellobiose; C, oligosaccharide plus enzyme, 0 min; D, 10 min; E, 30 min; F, 60 min; G, 120 min; H, 240 min.

the oligosaccharide were available for study, positive identification must await the isolation of larger amounts.

It should be noted that after the developed chromatograms stood for several hours traces of an unidentified substance appeared midway between glucose and cellobiose. This spot gave a green color reaction with benzidine rather than the characteristic brown of reducing sugars. Eluates of this substance gave a negative reaction with both the anthrone and the Somogyi reagent. This substance was also present in a hydrochloric acid hydrolyzate of cellulose.

The significance of this demonstration is that it gives form and substance to the random cleavage hypothesis (14). Further investigations and complete characterization of this and other intermediates are in progress.

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14. The investigation reported here was supported in part by a grant from the National Science Foundation.

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Hydrogen Bonding in 7-Ketocholesterol and a New Isomorph of 7-Ketocholesteryl Acetate

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In 1946 Furchgott, Rosenkrantz, and Shorr (1), employing infrared techniques, demonstrated that many steroids undergo hydrogen bonding in the solid state. This phenomenon has been observed, not only for the physiologically important steroids (1, 2), but also for ergostane compounds (3) and in the cholesterol series (4). In all cases one hydroxyl band occurred and the hydrogen bonding was evident because of displacement of the hydroxyl stretching vibrations from the unassociated wave number near 3640 cm^{-1} (wavelength, $2.75\text{ }\mu$) to wave numbers nearer 3330 cm^{-1} (wavelength, $3\text{ }\mu$).

During the infrared studies on the cholestane derivatives (4), it was observed that only 7-ketocholesterol gave rise to two absorption bands in the hydroxyl region. This occurred in the spectra of solid films, Nujol mulls, carbon disulfide (10 mg/ml, 1-mm cell) and carbon tetrachloride (5 mg/ml, 2-mm cell) solutions. In the solid state both bands were nearly of equal intensity, the sharper one occurring near 3545 cm^{-1} (wavelength $2.82\text{ }\mu$), while a broader one was located near 3280 cm^{-1} (wavelength $3.05\text{ }\mu$). In the solutions the usual increase in wave number owing to this state was observed, 3615 cm^{-1} (wavelength $2.77\text{ }\mu$) and 3417 cm^{-1} (wavelength $2.93\text{ }\mu$), respectively. No significant displacement of the ketone absorption occurred either in the solid state or in solution.

A doublet in the hydroxyl region was observed by Jones *et al.* (2) in the spectra of 17α -hydroxy-20-ketosteroids. Intramolecular hydrogen bonding was proposed as an explanation for the hydroxyl doublet, and it was reported that a threefold dilution in carbon tetrachloride did not alter the relative intensities of the two hydroxyl bands. Splitting of the normal 20-ketone absorption band was interpreted as indicative of an equilibrium between bonded and unbonded molecules. One other instance of the appearance of a doublet in the $3\text{-}\mu$ region has been reported for cholestane- $3\beta,5\alpha$ -diol-6-one (5).

Dilution of a solute involved in hydrogen bonding

is known to decrease the intensity of the associated absorption band and increase that of the higher frequency band. In the present study (6) a twofold or threefold dilution in carbon tetrachloride or carbon disulfide did not alter the relative intensities of the two hydroxyl bands. Before postulating hydrogen bonding for the origin of the $3\text{-}\mu$ doublet, the possibility of tautomerism was examined by attempting to synthesize the enol acetate of 7-ketocholesterol. On refluxing 7-ketocholesteryl acetate with acetic anhydride and acetyl chloride, $\Delta^{3,5}$ -cholestadiene-7-one (7) was obtained in 25-percent yield. The structure was established by infrared analysis, melting point 110° to 112°C , and elemental analysis: found C, 84.52; H, 10.90; calculated C, 84.75; H, 11.07. Treatment with acetic anhydride and sodium acetate or with acetic anhydride and pyridine resulted in formation of a dimorph (mp 163° to 164°C ; found C, 78.57; H, 10.41; calculated C, 78.68; H, 10.47) of the starting material (mp 153° to 155°C). The lower melting modification could be transformed into the higher melting form by seeding its pyridine solution with crystals of the new isomorph. Both compounds gave identical infrared spectra in carbon disulfide solution (10 mg/ml; 1-mm cell; 12 C Perkin-Elmer infrared spectrometer).

Ultraviolet spectroscopic analysis was also employed in an attempt to estimate quantitatively any possible enol structure. If 7-ketocholesteryl acetate enolized, then either a $\Delta^{5,7}$ -diene or a more stable $\Delta^{4,6}$ -diene would arise. The absorption of the latter arrangement cannot be distinguished from the $240\text{-m}\mu$ absorption of the parent molecule, but the $\Delta^{5,7}$ -structure absorbs near $285\text{ m}\mu$. Therefore, although variation in the density at $240\text{ m}\mu$ could not be interpreted, appearance of a maximum near $285\text{ m}\mu$ could favor the formation of the $\Delta^{5,7}$ -diene intermediate.

7-Ketocholesteryl acetate was studied in neutral (isooctane), acidic (acetic anhydride plus acetyl chloride) and basic (0.07*N* tetramethylammonium hydroxide in 90-percent ethanol) solutions [method of A. S. Meyer, personal communication] in a 1-cm cell at concentrations of $20\text{ }\mu\text{g/ml}$. Either a Beckman DU or Cary 11 MS spectrophotometer was used for the determinations at zero, 4 and 24 hr.

No absorption occurred under neutral or acidic conditions, while a maximum near $285\text{ m}\mu$ in the basic medium could account for approximately 20 percent intermediate. Whether this $285\text{-m}\mu$ maximum is related to the $\Delta^{5,7}$ -diene structure under the basic conditions used remains to be seen, but it should be recalled that enol acetate formation is catalyzed by acids.

Failure to prepare the enol acetate of 7-ketocholesterol by the usual chemical means in addition to the ultraviolet findings indicated that tautomerism did not significantly occur in this molecule. Therefore, the origin of the two absorption bands in the hydroxyl region have been assigned to an unusual tendency of intermolecular hydrogen bonding in 7-ketocholesterol. Indeed, this phenomenon may explain the difficulty in synthesizing the enol acetate of 7-ketocholesterol. Ad-