were observed: $-0.54°$ (1.3 min., 45 min.), and $-0.60°$ (200 and 400 min.). Attempts to recover crystalline material from the solution after 7 hr. were unsuccessful.

Anal. Calcd. for $C_{26}H_{22}O_8$: C, 67.52; H, 4.80. Found: C, 67.29; H, 4.97.

In another, larger-scale hydrolysis (which gave the same product in 27% yield) the material remaining in the mother liquor was treated with a mixture of acetic anhydride, acetic acid and hydrogen bromide. Subsequent hydrolysis afforded a second batch of 1,3,5-tri-*O*-benzoyl-β-D-arabinose and raised the total yield of this product to 39%.

β-D-Arabinofuranose Tetrabenzoate (X) from 1,3,5-Tri-*O*-benzoyl-β-D-arabinose (VIII).—A cold mixture of 1.0 ml. of dry pyridine and 0.20 ml. of benzoyl chloride was added to 68.1 mg. of 1,3,5-tri-*O*-benzoyl-β-D-arabinose. After 15 min. at 0° the reaction mixture was held at room temperature for one hour before the excess of benzoyl chloride was destroyed through the addition of a drop of water. Ten minutes later, much water was added, the product precipitating as a crystalline mass which was recrystallized from absolute ethanol: 55 mg. (66%), m.p. 120–122°, $[\alpha]^{20}D$ $-94.0°$ ($CHCl_3$, *c* 2.19). One further recrystallization from ethanol gave pure β-D-arabinofuranose tetrabenzoate melting at 121–122° and showing $[\alpha]^{20}D$ $-95.2°$ in chloroform (*c* 1.86).

Anal. Calcd. for $C_{33}H_{26}O_9$: C, 69.96; H, 4.63. Found: C, 70.12; H, 4.51.

2-*O*-Acetyl-1,3,5-tri-*O*-benzoyl-β-D-arabinose (XI).—A cold mixture of 1.0 ml. of dry pyridine and 0.20 ml. of acetic anhydride was added to 101.8 mg. of 1,3,5-tri-*O*-benzoyl-β-D-arabinose. After 45 min. at 0° and 17 hr. at room temperature excess of acetic anhydride was decomposed by the addition of water. The crystalline product thus obtained was washed with water, dried and recrystallized from 8 ml. of 1:1 absolute ethanol–pentane: 99.5 mg. (90%), m.p. 132–134°. Further recrystallization of the fine needles from ether did not raise this melting point. The pure 2-*O*-acetyl-1,3,5-tri-*O*-benzoyl-β-D-arabinose rotated $[\alpha]^{20}D$ $-60.4°$ in chloroform (*c* 1.91).

Anal. Calcd. for $C_{28}H_{24}O_9$: C, 66.66; H, 4.80. Found: C, 66.86; H, 4.84.

1,3,5-Tri-*O*-benzoyl-2-*O*-methylsulfonyl-β-D-arabinose (XII).—Crystalline 1,3,5-tri-*O*-benzoyl-β-D-arabinose (2.00 g.) was added portionwise over a period of 2 min. to a cold mixture of 4 ml. of dry pyridine and 0.4 ml. of methanesulfonyl chloride. After 1.2 hr. at room temperature the reaction mixture was diluted with methylene chloride and washed successively with 3 *N* sulfuric acid and saturated aqueous sodium bicarbonate. The solution was dried with sodium sulfate and concentrated *in vacuo* to a crystalline mass. Recrystallized from methylene chloride–ethanol the product (2.29 g., 98%) melted at 154–155° after sintering at 148°. One further recrystallization from ethyl acetate gave the pure ester showing a double melting point, 147°; 154–156° and rotating $[\alpha]^{20}D$ $-41.0°$ in chloroform (*c* 1.28).

Anal. Calcd. for $C_{27}H_{24}O_{10}S$: C, 59.99; H, 4.48; S, 5.93. Found: C, 59.71; H, 4.48; S, 5.91.

Treatment of 1,3,5-Tri-*O*-benzoyl-2-*O*-methylsulfonyl-β-D-arabinose (XII) with Sodium Methoxide.—To a suspension of the ester (1.80 g., 0.00333 mole) in 20 ml. of absolute methanol was added slowly 2.2 ml. of 1.3 *N* sodium methoxide. After 10 min. solution was complete and 1 ml. more of the sodium methoxide solution was added. Sodium methanesulfonate was observed to precipitate. A further 3.8 ml. of the sodium methoxide solution (to make a total of 0.009 mole) was added and the reaction mixture left at room temperature overnight. Two drops of water were added and carbon dioxide then passed in until the mixture was neutral. After removal of the precipitate the solution was concentrated *in vacuo* to a sirup which was dissolved in 30 ml. of water and extracted with methylene chloride to remove methyl benzoate. It was then passed successively through Amberlite IR-100 and Duolite A-4 to give a neutral solution which was concentrated *in vacuo* (60° bath) to a sirup. The mixture was then dissolved in ethanol, the solution clarified by filtration through Super-Cel and reconcentrated to a sirup (0.47 g.) which reduced Fehling solution. Ascending paper partition chromatography of a sample on Whatman #1 paper using 2-butanone saturated with water and Lemieux and Bauer's[15] $NaIO_4$-$KMnO_4$ spray reagent revealed five components which, relative to methyl β-D-ribopyranoside, had the following migration rates (averages from two determinations): 1.00, 0.83, 0.22, 0.09, 0.00. Simultaneous chromatography under identical conditions with known substances gave the following values: D-ribose, 0.20; D-arabinose, 0.08; D-lyxose, 0.12; methyl α-D-arabinopyranoside, 0.26; methyl α-D-arabinofuranoside, 0.94; methyl β-D-ribopyranoside, 1.00; methyl β-D-ribofuranoside, 0.84. Attempts to crystallize the sirup failed. A sample (10.03 g.) was dissolved in 2.5 ml. of *N* hydrochloric acid and heated on the steam-bath for 1.7 hr. Descending paper partition chromatography of the product in 3:2:1.5 1-butanol–pyridine–water revealed components with the following migration rates relative to ribose: 1.01, 0.74, 0.11, 0.00. Under the same conditions known substances gave values as follows: D-ribose, 1.00; D-arabinose, 0.73; D-lyxose, 0.94; D-xylose, 0.90. In the chromatogram of the reaction mixture the spot corresponding to D-arabinose was relatively weak compared with that corresponding to D-ribose.

A portion (0.43 g.) of the sirup was extracted with hot ethyl acetate and the extract concentrated to a sirup (0.38 g.) which was benzoylated in the usual manner using 3 ml. of dry pyridine and 1.0 ml. of benzoyl chloride. After the customary removal of reagents the resulting oil (1.12 g.) was chromatographed on alumina to give from ether–pentane 131.4 mg. of crystalline product which rotated $[\alpha]^{20}D$ $-70.7°$ ($CHCl_3$, *c* 2.02) and melted at 109–110° either alone or in admixture with authentic methyl β-D-ribopyranoside tribenzoate. The pure ester has been recorded[16] as melting at 109–110° and rotating $-69.5°$ ($CHCl_3$, *c* 0.82).

(15) R. U. Lemieux and H. F. Bauer, *Anal. Chem.*, **26**, 920 (1954).

(16) R. W. Jeanloz, H. G. Fletcher, Jr., and C. S. Hudson, THIS JOURNAL, **70**, 4055 (1948).

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[CONTRIBUTION FROM THE BIOCHEMICAL LABORATORY, THE DOW CHEMICAL COMPANY]

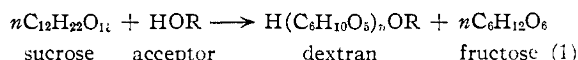
Studies on the Enzyme Dextransucrase. I. The Effect of *p*H on Enzyme Activity

BY W. BROCK NEELY

RECEIVED NOVEMBER 18, 1957

The Michaelis constant and maximum initial velocity for the enzymatic formation of dextran from sucrose were determined at various hydrogen ion concentrations. A mechanism is postulated for the above results. The scheme that is presented indicates a bifunctional catalytic role for the enzyme. The *pK* values of the active sites correspond to a carboxyl and an imidazole group.

In 1941–42 it was first demonstrated that cell free extracts of *Leuconostoc mesenteroides* were found to bring about the formation of dextran from sucrose.[1,2] By the action of the enzyme, sucrose is converted to dextran and one molecular equivalent of fructose.[1,2] The over-all reaction is represented in equation 1



$$nC_{12}H_{22}O_{11} + HOR \longrightarrow H(C_6H_{10}O_5)_nOR + nC_6H_{12}O_6 \quad (1)$$

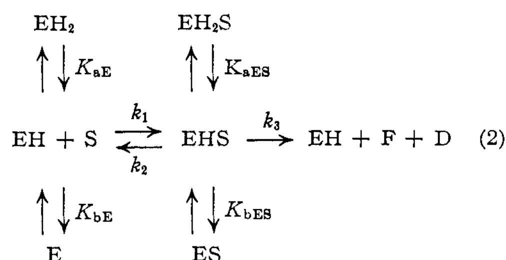
sucrose acceptor dextran fructose

(1) E. J. Hehre, *Science*, **93**, 237 (1941).

(2) E. J. Hehre and J. Y. Sugg, *J. Exptl. Med.*, **75**, 339 (1942).

The structure of dextran has been worked out by many investigators, and they have shown that the primary linkage is α-1 → 6 with various degrees of branching in the 1 → 4 and 1 → 3-positions. The mode of action of the enzyme, on the other hand has received very little attention.[3] Hehre[4] performed some initial experiments and he was able to demonstrate that the initial velocity followed the Michaelis-Menten equation.

The present work was undertaken in an effort to learn more about the active sites of the enzyme. The data to be presented in this article show the variation of maximal initial velocities and Michaelis constants at 30° with pH. The interpretation of the results is based on the suggestion by Michaelis and co-workers[5] and extended by Alberty and Frieden[6] that the bell-shaped activity curves result from ionization of functional groups in the enzyme. These ideas are expressed in the following mechanism (2) for the action of dextransucrase on sucrose at constant buffer concentration.



In this scheme the substrate, sucrose, is assumed to be un-ionized and the enzyme E is considered to exist in three ionized forms EH_2, E and EH, the latter being the reactive form. The products o the reaction are fructose F and dextran D.

Experimental

The preparative method of Tsuchiya, *et al.*, for dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F was used.[7] Since appreciable quantities of the enzyme were found in the extra-cellular fluid, centrifugation at 3500 r.p.m. in the cold for 1/2 hour gave a crude cell free extract.

Assay.—Hehre[4] demonstrated that measurement of either sucrose disappearance, dextran formation or fructose liberation gave identical results in the estimation of dextransucrase activity (see equation 1). The measurement of fructose liberation was used in the present work. The technique for the assay is described by Tsuchiya, Koepsell, *et al.*[7,8] Fructose was measured by the Somogyi[9] method as modified by Nelson.[10]

Kinetics.—Kinetic measurements were carried out in 0.05 M Tris-maleate[11] buffer between pH 5.2–8.2. A 0.05 M acetate buffer was used between pH 4.5–5.2. The crude cell free extract solution assaying 35 units per ml.[12] was diluted 1 to 5 with the appropriate buffer. The reaction mixture consisted of 0.2 ml. of enzyme solution and 0.8 ml. of sucrose solution varying from 80 to 20 mM in a buffer similar to the enzyme solution. This reacted for 1 hour in a water-bath maintained at 30°. At the conclusion the reaction was quenched with 1 ml. of 0.08 N NaOH. The sugar analysis was carried out as described previously.[9,10] Michaelis constants and maximal initial velocities were determined by the graphical method of Lineweaver and Burk.[13]

Results

The presence of activators or inhibitors in the crude enzyme solution can be determined if varying dilutions of the preparation are tested against a fixed concentration of substrate. A plot of the initial velocity against the enzyme concentration will yield a straight line through the origin, if neither activator nor inhibitor is present.[14] Such a plot is shown in Fig. 1. The linearity of the graph indicates the freedom of the enzyme solution from activators or inhibitors in the concentration range that was examined.

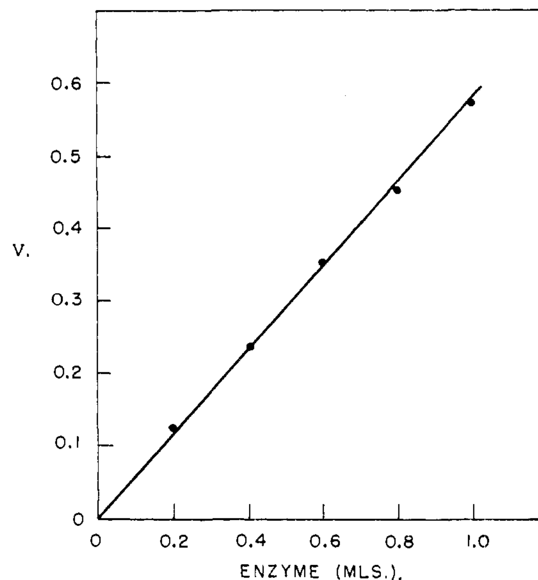


Fig. 1.—Plot of initial velocity (mg. fructose/1.4 ml./hour) against enzyme concentration. Reaction mixture consisted of 0.4 ml. of 100 mM sucrose, varying enzyme concentrations and 0.05 M buffer of pH 5.2 to bring the volume to 1.4 ml.

The steady-state treatment of mechanism (2) yields the Michaelis-Menten equation $v = V/(1 + K_M S)$ where V and K_M vary with pH according to the equations[6]

$$V = \frac{k_3(E)_0}{1 + (H^+)/K_{aES} + K_{bES}/(H^+)} \quad (3)$$

$$K_M = \frac{(k_2 + k_3)}{k_1} \frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K_{aES} + K_{bES}/(H^+)} \quad (4)$$

The values of pK_{aES} and pK_{bES} may be evaluated by the use of the following equations.[15] In

$$K_{aES} = (H^+)_a + (H^+)_b - 4[(H^+)_a(H^+)_b]^{1/2}$$

$$K_{bES} = \frac{(H^+)_a(H^+)_b}{K_{aES}} \quad (5)$$

these equations $(H^+)_a$ and $(H^+)_b$ are the hydrogen ion concentrations at which V is one-half the value

(3) J. Edelman, *Adv. Enzymol.*, **17**, 189 (1956).
(4) E. J. Hehre, *J. Biol. Chem.*, **163**, 221 (1946).
(5) (a) L. Michaelis and H. Davidson. *Biochem. Z.*, **35**, 386 (1911); (b) L. Michaelis and H. Pechstein, *ibid.*, **59**, 77 (1914).
(6) C. Frieden and R. A. Alberty, *J. Biol. Chem.*, **212**, 859 (1955).
(7) H. M. Tsuchiya, H. J. Koepsell, J. Corman, G. Bryant, M. O. Bogard, V. H. Feger and R. W. Jackson, *J. Bacteriol.*, **64**, 521 (1952).
(8) H. J. Koepsell and H. M. Tsuchiya. *ibid.*, **63**, 293 (1952).
(9) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).
(10) N. Nelson, *ibid.*, **153**, 375 (1944).
(11) Tris-(hydroxymethyl)-aminomethane maleate.
(12) The dextransucrase unit is defined as the amount of enzyme which will convert 1 mg. of sucrose to dextran in 1 hour (releasing 0.52 mg. of fructose) under standard conditions.
(13) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).
(14) J. S. Friedenwald and G. D. Maengwyn-Davies, "The Mechanism of Enzyme Action," W. B. McElroy and B. Glass, eds., The Johns Hopkins Press, Baltimore, Md., 1954, p. 174.
(15) R. A. Alberty and V. Massey, *Biochim. et Biophys. Acta*, **13**, 347 (1954).

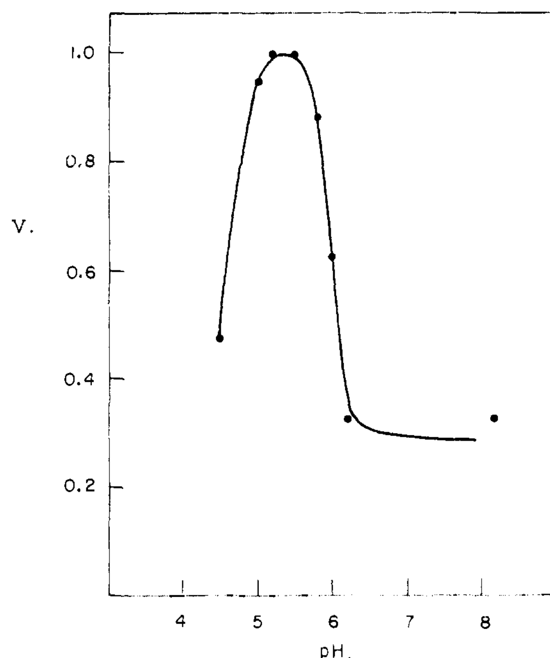


Fig. 2.—Plot of maximum initial velocity (V) against pH.

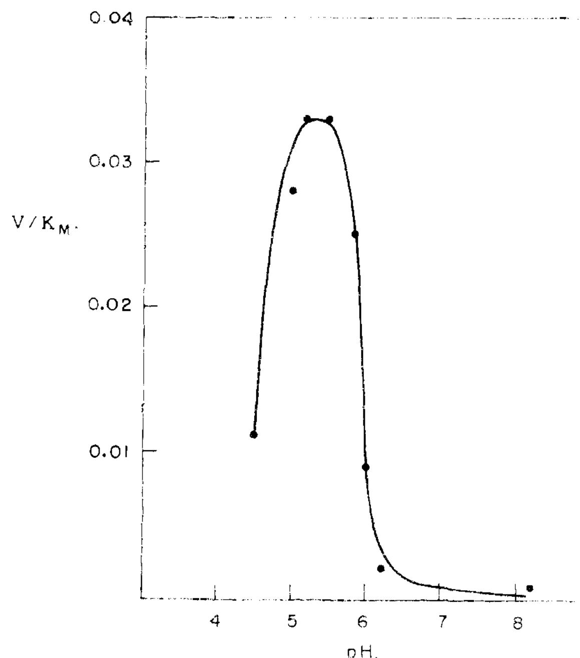


Fig. 3.—Plot of maximum initial velocity over Michaelis constant (V/K_M) against pH.

at optimum pH, determined from a plot of V against pH (Fig. 2).

The denominators in equations 3 and 4 are similar, consequently, V/K_M becomes a function of H^+, K_{aE} and K_{bE}. The values of these latter two constants may then be evaluated from a plot of V/K_M against pH (Fig. 3) and the subsequent use of equations 5.[14]

The calculated values for the various dissociation constants are given in Table I.

TABLE I

VALUES OF THE DISSOCIATION CONSTANTS OF THE ENZYME AND ENZYME SUBSTRATE

	E (Fig. 3)	ES (Fig. 2)
pK_a	5.1	4.9
pK_b	5.5	5.7

The results of the determination of maximal initial velocities and Michaelis constants are shown in Table II.

TABLE II

VARIATION OF MAXIMAL INITIAL VELOCITIES AND MICHAELIS CONSTANTS WITH pH AT 30°

pH	K_M, mM	V^a
4.5	42	0.47
5.0	34	0.95
5.2	30	1.0
5.5	30	1.0
5.8	35	0.87
6.0	82	.63
6.2	160	.33
8.2	300[b]	.33[b]

[a] V = mg. fructose/0.2 ml./hour. [b] These values are questionable as they are at the limit of the analytical sensitivity.

The optimum pH for the enzyme is 5.2–5.5 and the Michaelis constant 30 mM agrees fairly well with Hehre's value of 20 mM[4] when it is considered that this investigator made his determination at 23°.

Discussion

Of the polar side groups present in the protein portion of dextransucrase which might act as catalytic sites, the carboxyl and imidazole groups appear to fit the observed data. The nucleophilic group is undoubtedly a carboxyl as no other groups in the protein have a pK value corresponding to 4.9–5.1. The observed pK for the electrophilic group is slightly low for an imidazole group. However, as Gutfreund[16] has stated the pK of the imidazole group of histidine in proteins and peptides has been reported to have values ranging from 5.5 to 7.0. The value obtained in the present work falls within this range. Sulfhydryl groups may be eliminated as a possibility since it was shown that iodoacetate had no effect on dextransucrase.[4] The guanidinium, hydroxyl and amino groups present as side groups in the protein molecule are too basic to be considered. The implication of the imidazole group is most interesting since it has been shown to be involved with the catalytic site of a large number of enzymes. Thus from studies on the pH dependence of enzymatic activity and the loss of activity upon the photooxidation of histidine, it has been inferred that intestinal carbohydrases,[17] chymotrypsin,[18,19] trypsin,[16] acetylcholine esterase,[20] lysozyme[21] and ribonuclease[22] owe part of their activity to the presence of intact imidazole groups in the protein molecule.

(16) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955).
(17) J. Larner and R. E. Gillespie, *Arch. Biochem. Biophys.*, **58**, 252 (1955).
(18) R. B. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955).
(19) L. Weil and A. R. Buchert, *Federation Proc.*, **11**, 307 (1952).
(20) I. B. Wilson and F. Bergmann, *J. Biol. Chem*, **186**, 683 (1950).
(21) L. Weil, A. R. Buchert and J. Maber, *Arch. Biochem. Biophys.*, **40**, 245 (1952).
(22) L. Weil and T. S. Seibles, *ibid.*, **54**, 368 (1955).

A reasonable mechanism for the polymerization action of dextransucrase would be a termolecular process as postulated by Swain.[23] This author[23] was able to amplify an earlier suggestion by Lowry and Faulkner,[24] that in mutarotation α-D-glucose was subjected to a simultaneous attack by a nucleophilic and an electrophilic group. Later[25] it was demonstrated that of a series of hydroxypyridines only one the 2-hydroxypyridine contained the nucleophilic and electrophilic groups in the correct spatial arrangement for optimum catalysis of the mutarotation of tetra-*O*-methyl-α-D-glucopyranose. Such a situation might exist in dextransucrase where an imidazole and a carboxyl group are so situated in the protein matrix that they can effect a concerted attack on the glycosidic linkage of sucrose liberating a glucosyl group for the polymerization reaction. In this mechanism the rate-determining step would be the cleavage of the glycosidic linkage of the substrate.

The substrate specificity for dextransucrase is very rigid in that only sucrose has been shown to act as a glucosyl donor. It could be assumed, therefore, that apart from combination of the enzyme and substrate at a catalytic site, there must also be binding at a specificity site. Work is in progress to determine more about the nature of these two sites for this particular enzyme.

Acknowledgment.—The author is indebted to Dr. D. Dyer of this Laboratory for his advice and assistance in producing the enzyme.

MIDLAND, MICHIGAN

(23) C. G. Swain, THIS JOURNAL, **72**, 4578 (1950).
(24) T. M. Lowry and I. J. Faulkner, *J. Chem. Soc.*, **127**, 2883 (1925).
(25) C. G. Swain and J. F. Brown, THIS JOURNAL, **74**, 2538 (1952).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, THE UNIVERSITY OF ROCHESTER, SCHOOL OF MEDICINE AND DENTISTRY]

Composition and Properties of the Thymus Desoxyribonucleoprotein of Doty and Zubay[1]

BY ALEXANDER L. DOUNCE AND MARGUERITE O'CONNELL

RECEIVED JULY 10, 1957

In a recent communication to THIS JOURNAL, Doty and Zubay[2] reported the isolation of a nucleoprotein from calf thymus which contained approximately equal amounts of desoxyribonucleic acid (DNA) and protein, and which was thought possibly to be the principal structural component of chromosomes. Physiochemical studies of this material were reported. It was stated that gel formation by the nucleoprotein could be avoided by treating the material as specified in the communication.

In this Laboratory, however, we consider gel formation to be an indication of an intact desoxyribonucleoprotein, where the DNA is attached to the residual lipoprotein of the chromosomes, probably through covalent bonds.[3] Moreover it seemed likely to us that the method of isolation used by Doty and Zubay[1] would yield material approximating the isolated chromosomes of Mirsky in composition.[4-6]

We have therefore repeated the isolation of the material of Doty and Zubay[2] and have subjected this to analysis. In order to facilitate storage the material was dried from solution by the process of lyophilization. RNA and DNA were extracted from these samples with hot 5% TCA and determined by the method of Schneider, using the orcinol and diphenylamine reactions for color formation. Globulins were removed in cold 0.9% saline and precipitated with saturated ammonium sulfate. Water-soluble material (mainly inorganic) was determined by dissolving samples of the lyophilized nucleoprotein in water, drying at 105° and carefully washing the dried samples of the nucleoprotein with distilled water and drying them again at 105° for nine hours until the weight loss became negligible. Lipid was extracted from aliquots of the nucleoprotein in 25% ether–75% ethanol with a continuous Soxhlet extractor and then was isolated by dissolving in 30–60° petroleum ether. The material soluble in petroleum ether was determined by weighing after drying at 105° for nine hours. Histone was extracted from the lipid-free material with cold 0.2 *N* HCl and precipitated with concentrated NH_4OH on standing nine hours in the cold. The alcohol-precipitable histone was precipitated from the supernatant fluid by adding 3 volumes of 95% ethanol and allowing to stand for 2 to 3 days in the cold. The two histone samples were dried in the oven at 105°, carefully washed with distilled water to remove any salt and then redried at 105° before weighing. The residual protein was determined by first weighing the residue from lipid-extracted, histone-extracted material, after careful washing and drying to remove any salt that might be present. Then the weight contributed to the sample by RNA plus DNA was calculated and subtracted to give the weight of the residual protein. We find the composition to be approximately as given in Table I. Thus the material does in fact resemble Mirsky's isolated chromosomes in chemical composition.

The data that have been recalculated on the basis of material free of the water-soluble fraction

(1) We gratefully acknowledge the support of The National Cancer Institute, U. S. Public Health Service, Grant No. C-994, which has made this work possible.
(2) P. Doty and G. Zubay, THIS JOURNAL, **78**, 6207 (1956).
(3) (a) A. L. Dounce and K. J. Monty, *J. Biophys. Biochem. Cytol.*, **1**, 155 (1955). (b) K. J. Monty and A. L. Dounce, *J. Gen. Physiol.*, **41**, 595 (1958).
(4) A. E. Mirsky and A. W. Pollister, *ibid.*, **30**, 117 (1946).
(5) A. E. Mirsky and H. Ris, *ibid.*, **31**, 7 (1947).
(6) A. E. Mirsky and H. Ris, *ibid.*, **34**, 475 (1951).