which had the properties of "α-sphingosine sulfate,"[6] *i.e.*, it gradually became yellow on exposure to air, and upon irradiation with ultraviolet light (Hanovia utility lamp) specimens exhibited a whitish fluorescence whose intensity considerably exceeded that of a sample of quinine sulfate (violet fluorescence) which was examined under comparable conditions.

Anal. Calcd. for $(C_{18}H_{37}O_2N)_2 \cdot H_2SO_4$: C, 62.0; H, 10.99; N, 4.02. Found: C, 61.4; H, 10.95; N, 3.83.

A sample of sphingosine, freshly prepared *via* the acid hydrolysis of beef cord, was kindly supplied by Dr. Irving Zabin, Physiol. Chem. Dept., the Medical School, U.C.L.A., Los Angeles, Calif. The sample, recrystallized from acetone, had m.p. 96°.

Triacetylsphingosine.—This compound was prepared from sphingosine sulfate according to the directions of Carter and co-workers[24] and purified by recrystallization from acetone. The material thus obtained had $[\alpha]^{27}D$ −10.9° (*c* 1.8, chloroform), m.p. 101–102° (repd.[24] $[\alpha]^{25}D$ −11.7° (chloroform), m.p. 101–102°).

Anal. Calcd. for $C_{24}H_{43}O_5N$: C, 67.7; H, 10.18; N, 3.29. Found: C, 67.5; H, 10.25; N, 3.02.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY]

On the Cytosine Deaminase of Yeast[1]

BY JACOB KREAM AND ERWIN CHARGAFF

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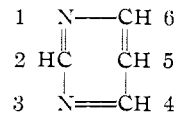
The description of the isolation of partially purified, stable cytosine deaminase preparations from bakers' yeast is followed by a study of the properties of the enzyme. It degrades cytosine with the production of equimolar quantities of uracil and ammonia in a unimolecular reaction at a *p*H optimum of 6.9. The effects of enzyme and substrate concentration and of temperature on the course of the enzymatic action have been investigated. The Michaelis constant has been found as 8.4×10^{-3} *M*, the temperature velocity constant as 19,500 cal./mole. Of many other pyrimidines studied, only 5-methylcytosine was converted to thymine by the enzyme preparations. Isocytosine acted as an inhibitor. Conclusions as to the specificity requirements of cytosine deaminase are discussed, and a procedure for the chromatographic separation of 5-methylcytosine from other pyrimidines and for its quantitative determination in minute amounts is described.

The present paper considers the cytosine deaminase of yeast in some detail. Brief accounts of some of the phases of the work have appeared.[2,3] Enzyme systems capable of deaminating cytosine to uracil have been encountered rather infrequently. Extracts of animal tissues appear in general to be unable to degrade cytosine itself, although the ribonucleoside cytidine is attacked.[4,5] That dietary cytosine can be converted to uracil in the body has been both denied[6] and affirmed.[7] Indications of the production of ammonia from cytosine, incubated with fowl blood, have been reported.[8]

The presence in bakers' yeast of enzymes able to deaminate cytosine and 5-methylcytosine to uracil and thymine, respectively, is well established.[2,3,9,10] A cytosine deaminase also has been found in *E. coli*[2,11,12] and obtained as a cell-free extract[12]; but this property does not seem to be inherent in all strains.[13] The development of convenient micro procedures for the study of purine and pyrimidine deaminases and the discovery that yeast was a good source for the isolation of cell-free preparations of cytosine deaminase[2,12] prompted a more detailed investigation of this enzyme, also with respect to its specificity characteristics.

Experimental

Material.—The following numbering system will be used in describing the pyrimidines.



Uracil either was used as a purified commercial preparation or synthesized here.[14] Cytosine was prepared from uracil,[15] as was 2-methoxy-6-aminopyrimidine. 5-Methylcytosine and 2-mercapto-6-aminopyrimidine were obtained through the courtesy of Dr. G. H. Hitchings of the Wellcome Research Laboratories, Tuckahoe, N. Y. For 2,6-diaminopyrimidine, 2-hydroxy-4,6-diaminopyrimidine and isoguanine (2-hydroxy-6-aminopurine) we are highly indebted to Dr. A. Bendich of the Sloan-Kettering Institute for Cancer Research, New York; for isocytosine (2-amino-6-hydroxypyrimidine) and 2,6-dihydroxy-5-aminopyrimidine to Dr. M. Hoffer of Hoffmann-La Roche, Inc., Nutley, N.J.; for 4-hydroxycytosine to Dr. M. Engelman of this college. The preparation of cytidylic acid has been described previously.[16] Cytidine was a purified commercial preparation.

Quantitative Determinations.—Cytosine and uracil were separated by filter paper chromatography and estimated by spectrophotometry, as described in a previous publication.[17] Essentially similar procedures served for the separation of thymine from 5-methylcytosine and the quantitative determination of the latter. For the techniques used, the recently published procedures for the estimation of xanthine and hypoxanthine should also be consulted.[12] The separa-

(1) Supported by a research grant from the National Institutes of Health, United States Public Health Service. This report is based on a dissertation submitted by Jacob Kream in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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TABLE I

DEAMINATION OF CYTOSINE BY YEAST CYTOSINE DEAMINASE[a]

Incubation time, min.	Cytosine remaining: Experiment no. 1 γ	2 γ	3 γ	Average γ	Average Micromoles	Uracil formed: Experiment no. 1 γ	2 γ	3 γ	Average γ	Average Micromoles	Total pyrimidine accounted for, %	Extent of deamination, %
15	92.8	95.2	101	96.3	0.87	14.2	14.5	14.5	14.4	0.13	93	12
30	89.0	84.7	86.2	86.6	.78	26.6	25.2	25.6	25.8	.23	94	21
45	78.0	80.4	78.0	78.8	.71	34.4	34.5	33.7	34.2	.30	94	28
60	74.6	73.6	74.8	74.3	.67	39.6	39.0	39.0	39.2	.35	95	32
90	68.2	67.2	68.8	68.1	.61	46.4	44.6	46.2	45.7	.41	95	38
120	66.2	65.5	65.2	65.6	.59	48.0	48.8	49.4	48.7	.43	95	40

[a] A mixture of 2 ml. of a 0.25% aqueous enzyme solution and 2 ml. of a 0.108 *M* cytosine solution in 0.4 *M* phosphate buffer of *p*H 6.9 was incubated at 37.5°. Each experiment corresponds to a 0.02-ml. aliquot (containing a total of 1.08 micromoles of cytosine at zero time).

tions were carried out with *n*-butanol saturated with water as the solvent system, followed by the location of the separated zones under an ultraviolet lamp. The relative R_F values, with that of thymine taken as 100, were: cytosine 42, 5-methylcytosine 54, uracil 69. Phosphate buffer (0.01 *M*) of *p*H 7.0 served both as the solvent of the test mixtures and for the extraction of the separated zones. Under these conditions, authentic 5-methylcytosine had its absorption maximum at 274 mμ with ε 6050. Following the principles outlined before,[17] the extracts were read at 300, 279 and 269 mμ, in order to exclude contaminated solutions and to define the shape of the spectrum, and at 274 and 295 mμ, the differences Δ between the extinctions *E* at those two points serving for the calculation of the content. For 10 γ of 5-methylcytosine per ml. the following values were found: E_{274} 0.483; E_{295} 0.079; Δ = 0.404. In a series of determinations in which between 5 and 10 γ of 5-methylcytosine were subjected to chromatography, in some cases in the presence of 15 γ of thymine, the average percentage of 5-methylcytosine recovered was 100, with a standard deviation of 1.[18] The quantitative determination of thymine already has been described.[17]

Procedures.—The methods used have been discussed in a recent publication.[12] In the present study, the incubation was always carried out *in vitro*, and the reaction products then were assayed on filter paper as described there. The purification of the enzyme was followed with a 45 m*M* solution of cytosine in 0.1 *M* phosphate buffer of *p*H 7.0; the partially purified enzyme preparations were studied with 0.108 *M* cytosine solutions in 0.4 *M* phosphate buffer of *p*H 6.9 as the substrate. Similar solutions were employed with the other pyrimidines, which were screened for susceptibility to enzymatic deamination by means of micro determination of NH_3.[19] Before aliquots of the incubation mixtures were subjected to chromatography, the enzymatic action was stopped either by the acidification of the aliquots before dispensing them on paper or by the subsequent heating of the strips to 90–100° for 15 minutes[12]; the results were identical. All values reported here represent the average of at least 3 parallel chromatographic determinations. The protein contents of the enzyme solutions were estimated by ultraviolet spectroscopy.[20,21]

Preparation of Enzyme.—All operations were carried out in the cold. In a typical preparation, 50-ml. portions of a suspension of 454 g. of bakers' yeast in 80 ml. of dist. water were passed for 30 minutes through the Unicam wet crushing mill for bacteria,[22] and the mixture, diluted with an equal volume of dist. water, was centrifuged for 1 hour at 1900 × *g*, when two sediment layers were deposited beneath an opalescent supernatant (650 ml.). About 60% of the yeast cells had been crushed. The clear yellow solution, resulting from the centrifugation of the turbid supernatant at 15,000 × *g* for 2 hours, was adjusted to 70% saturation by the addition of solid ammonium sulfate. Following storage overnight at −15°, the precipitate thus produced, which contained the entire cytosine deaminase activity, was collected by centrifugation at 1900 × *g*, washed twice by suspension in a $(NH_4)_2SO_4$ solution of the same concentration, dissolved in 100 ml. of water and dialyzed, with rocking, against running cold tap water and several changes of ice-cold dist. water for 24 hours, until the outside fluid gave no Nessler reaction for ammonia. The solution was brought to 25% $(NH_4)_2SO_4$ saturation and the precipitate centrifuged off and discarded. Adjustment of the supernatant to 70% $(NH_4)_2SO_4$ saturation resulted in a precipitate which was collected, washed and, after solution in 15 ml. of water, dialyzed as described above. The lyophilization of the dialyzed solution afforded 1.7 g. of a white fluff which consisted entirely of protein and was easily soluble in dist. water. Preparations of this type, which were used in all subsequent experiments, retained about 37% of the cytosine deaminase activity of the original supernatant resulting from the centrifugation at 1900 × *g* of the suspension of the crushed yeast cells. Based on the protein content, the purification was fourfold; on the dry weight, sixfold. The dry products were stored at −15°. Attempts at further fractionation within a narrower range of $(NH_4)_2SO_4$ concentration or by the use of ethanol at different concentrations failed to yield more active preparations.

Properties of Cytosine Deaminase.—Dry preparations, kept at −15°, retained their full activity for years; frozen aqueous solutions similarly stored also remained active for several months. Prolonged dialysis did not diminish the activity, nor did Mg ions increase it. The enzyme was, however, inactivated by heating at 80° for 15 minutes.

The course of conversion of cytosine to uracil by the purified enzyme is summarized in Table I. When these data are plotted in the usual fashion, it will be seen that the deamination of cytosine follows the course of a first order reaction, at least during the first 45 minutes of deamination, *i.e.*, until 28% of the substrate have been removed. A separate series of experiments showed that the production of uracil was accompanied by the liberation of almost equimolar quantities of ammonia (Table II). It will be noticed that borate buffer could be substituted for the phosphate buffer with no change in results. The effect of *p*H on enzymatic activity is illustrated in Fig. 1; a well-defined *p*H optimum at *p*H 6.9 was found under the conditions of

TABLE II

AMMONIA PRODUCTION DURING ENZYMATIC CYTOSINE DEAMINATION[a]

Incubation time, minutes	Uracil formed, micromoles	Ammonia formed, micromoles	Uracil to ammonia ratio
30	23	19	1.2
30	19[b]		
60	36	33	1.1

[a] In each experiment 1 ml. of a 0.25% aqueous enzyme solution and 1 ml. of a 0.108 *M* cytosine solution in 0.4 *M* phosphate buffer of *p*H 6.9 were incubated at 37.5°. Figures refer to the totals found in the reaction mixtures. No ammonia was produced in enzyme blanks. [b] In 0.2 *M* boric acid–sodium tetraborate buffer of *p*H 7.0.

(18) As a supplement to the previous demonstration of the stability of cytosine toward heating with cond. formic acid, which formed the basis of a hydrolysis procedure for nucleic acids,[16] it may be of interest to note that 5-methylcytosine has been found equally resistant and could be recovered quantitatively after being heated in a bomb tube for two hours at 175° in 98–100% formic acid.

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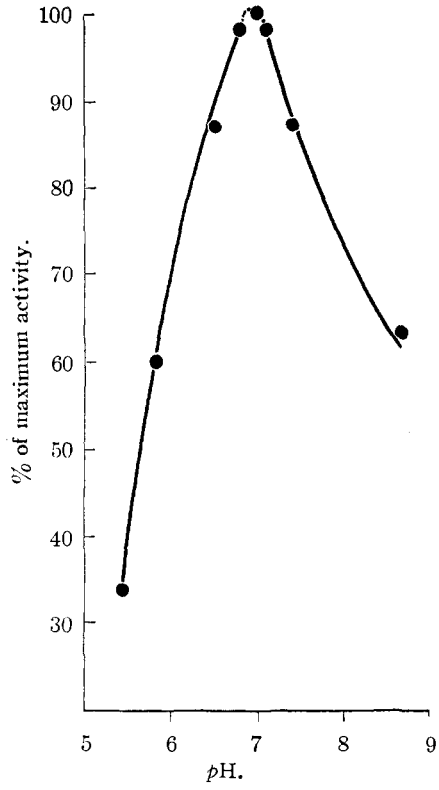

Fig. 1.—Effect of hydrogen ion concentration on extent of uracil production from cytosine by cytosine deaminase in 0.2 *M* phosphate buffers. Other conditions as in Table I.

this experiment. No disappearance of uracil was observed when this pyrimidine was incubated with the enzyme at *p*H 6.9 or 8; this is also borne out by the satisfactory recoveries of total pyrimidine recorded in Table I. The proportionality between enzyme concentration and extent of cytosine deamination is demonstrated in Table III. The effect of increasing the initial substrate concentration with a constant quantity of enzyme is shown in Fig. 2 for incubation periods of 15 and 60 minutes (curves A and B, respectively). No decrease in enzymatic activity was observed at and above the level of saturating substrate concentration (Fig. 2B), nor did a separate series of experiments, not reported here in detail, reveal a poisoning effect of the reaction products uracil and ammonia on the course of deamination. The drop in the rate of cytosine breakdown, observed when the incubation period was prolonged beyond about 60 minutes (Table I), doubtless was due to the gradual inactivation of the enzyme on prolonged exposure to 37.5° (compare Table IV).

TABLE III

EFFECT OF ENZYME CONCENTRATION ON CYTOSINE DEAMINATION[a]

Enzyme, mg.	Uracil formed during incubation period, micromoles 30 min.	60 min.
2.5	25	39
1.25	12	18
0.63	6	8

[a] Conditions as in Table I. All figures refer to total amounts in reaction mixture; the initial cytosine concentration was 108 micromoles.

TABLE IV

PARTIAL INACTIVATION OF CYTOSINE DEAMINASE SOLUTIONS BY EXPOSURE TO 37.5° IN THE ABSENCE OF SUBSTRATE[a]

Duration of exposure, minutes	Deamination, % Unbuffered solution (*p*H 5.6)	Buffered solution (*p*H 6.9)
0	35	35
20	12	24
60	4	20

[a] One-ml. aliquots of the 0.25% enzyme solution in dist. water (*p*H 5.6) or 0.2 *M* phosphate buffer (*p*H 6.9) were kept at 37.5° for the stated periods; 12 mg. of cytosine in 1 ml. of phosphate buffer or water, respectively, then was added and the incubation continued for 60 minutes. The extent of deamination was estimated from the values for uracil found at the end of the experiments.

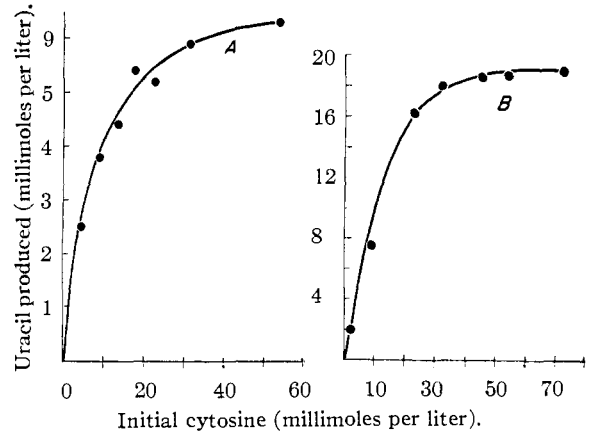

Fig. 2.—Uracil production by a constant quantity of cytosine deaminase at different levels of substrate concentration; incubation periods 15 minutes (curve A) and 60 minutes (curve B). Other conditions as in Table I.

The measurements reproduced in curve A of Fig. 2 were plotted according to Lineweaver and Burk,[23] with the quotient of substrate concentration and velocity as the ordinate and substrate concentration as the abscissa. From the statistically determined values for intercept and slope of the straight line the maximum value of the velocity was found as $V_{max} = 5 \times 10^{-4}$ *M* per minute and the Michaelis constant as $K_m = 8.4 \times 10^{-3}$ *M*. The alternative method[23] of plotting the reciprocal of velocity against the reciprocal of substrate concentration resulted in a figure of $K_m = 8.2 \times 10^{-3}$ *M*.

As is shown in Table IV, a considerable loss in activity was observed, when an unbuffered aqueous solution of the enzyme (*p*H 5.6) was kept at 37.5° in the absence of substrate. Inactivation, though less pronounced, also took place in solutions buffered at *p*H 6.9. The effect of varying the temperature of incubation of enzyme–substrate mixtures is illustrated in Fig. 3. For an incubation period of 60 minutes the temperature optimum was near 34°; for an incubation lasting 30 minutes it shifted to 37.5°. Values for the temperature coefficient Q_{10}, calculated for the range of 20–30°, within which no heat inactivation of the enzyme took place, were 2.9, based on uracil production, and 2.6, when the liberation of ammonia was followed. The graphical evaluation of the data presented in Fig. 3 yielded a straight line up to a temperature of 34.5° when inactivation of the enzyme begins; the temperature velocity constant was found as $\mu = 19{,}500$ cal./mole.

Deamination of 5-Methylcytosine.—The cytosine deaminase preparations from yeast attacked 5-methylcytosine vigorously and converted it to thymine. The averages of 2 series of experiments in which relatively small amounts of 5-methylcytosine were deaminated almost completely are presented in Table V. It should be mentioned that by the application of this enzymatic deamination and of the other techniques described in the present paper and in previous publications[16,17] it has been possible to study a minute quantity of a new desoxyribonucleotide, isolated from calf thymus desoxyribonucleic acid,[24] and, in collaboration with Dr. W. E. Cohn of the Oak Ridge National Laboratory, to establish its structure as desoxy-5-methylcytidylic acid.[25]

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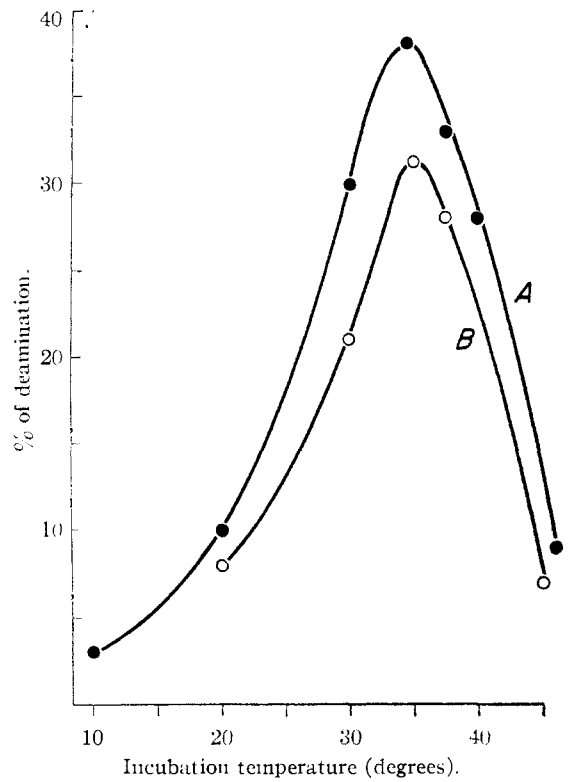

Fig. 3.—Effect of temperature of incubation on enzymatic deamination of cytosine; uracil production (curve A), ammonia liberation (curve B). Other conditions as in Table I.

TABLE V

DEAMINATION OF 5-METHYLCYTOSINE BY YEAST CYTOSINE DEAMINASE[a]

Incubation time, minutes	5-Methylcytosine remaining		Thymine formed		Total pyrimidine accounted for, %	Extent of deamination, %
	γ	micromoles	γ	micromoles		
60	6.5	0.05	34.2	0.27	97	82
120	6.5	.05	34.9	.28	100	85

[a] In each assay, 1 ml. of a 0.25% aqueous enzyme solution and 1 ml. of a 33 m*M* solution of 5-methylcytosine in 0.4 *M* phosphate buffer of *p*H 6.9 were incubated at 37.5°. The values reported refer to 0.02-ml. aliquots of the mixtures (corresponding to 0.33 micromole of initial substrate) and represent averages of 3 determinations.

Resistant and Inhibitory Compounds.—None of the other pyrimidines and analogs listed in the first section of the Experimental part was deaminated by the enzyme. 2-Amino-6-hydroxypyrimidine (isocytosine) and 2-mercapto-6-aminopyrimidine depressed the rate of cytosine deamination. For instance, when ammonia production from 108 micromoles of cytosine by 2.5 mg. of enzyme in a total volume of 2 ml. of phosphate buffer (60 minutes, 37.5°) was studied in the presence of varying amounts of isocytosine, the following effects were observed: 54 micromoles of isocytosine, 16% inhibition; 108 micromoles, 35%; 159 micromoles, 44%; 245 micromoles, 56%.[26]

Discussion

The enzymatic conversion of cytosine and 5-methylcytosine to uracil and thymine, respectively, by the partially purified deaminase preparation from yeast appears to be due to a hydrolytic deamination. It is accompanied by the liberation of equimolar quantities of ammonia. Atmospheric oxygen is not required,[12] nor have any indications been obtained of the production of a compound with the spectral characteristics[27] of 2-hydroxypyrimidine, as could have been expected as the result of a reductive deamination of cytosine. The establishment of complete balances (compare Tables I and V), to which the chromatographic technique employed here lends itself particularly well, excludes the presence in the yeast preparations of an enzyme of the type of the recently described uracil oxidase.[28] Whether cytosine and 5-methylcytosine are attacked by the same yeast enzyme, cannot yet be stated. Recent evidence against this identity[29] appears, however, inconclusive.

The failure of cytosine deaminase to attack some of the compounds tested in the course of this work permits certain conclusions with respect to the specificity characteristics of the enzyme. An unsubstituted hydroxyl in position 2 seems to be required; *i.e.*, cytosine is attacked in the lactim form. Substitution of this hydroxyl or its replacement by another group, such as thio or amino, renders the compounds not susceptible to enzymatic attack, as does also tautomerization to the lactam form; the introduction of a substituent in position 3 protects the substances from degradation. The intactness of the CH group in position 4 of cytosine also seems to be essential: 4-hydroxycytosine, 4-aminocytosine or isoguanine are not attacked. If 5-methylcytosine and cytosine are treated as substrates by the same enzyme, position 5 of cytosine may be considered as not essential for the action of the deaminase. Finally, the amino group must be situated in position 6: isocytosine is not deaminated by the yeast enzyme; it acts as an inhibitor.

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