[CONTRIBUTION FROM THE RESEARCH DEPARTMENT, THE ARMOUR LABORATORIES]

Ionic Inhibition of Bacterial Growth. II. Relationship of Ionic Inhibition to DNA-Protein Biosynthesis[1]

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The vitamin B_{12} (or thymidine) requirement of *Lactobacillus leichmannii* 313 is a function of the ionic strength of the growth medium between certain limits of concentration. Quantitative studies presented in this paper are consistent with the hypothesis that vitamin B_{12} combines with an "apoenzyme," E_a, to form the catalytic factor required for the growth of this organism. The concentration *vs.* ionic strength curve of E_a is similar to the solubility *vs.* ionic strength curve of DNA-protein. This observation points to the possibility that E_a is a DNA-protein, and that it acts as an autocatalyst in the biosynthesis of new DNA-protein.

Introduction

In an earlier communication from this Laboratory,[2] it has been reported that addition of various inorganic salts to a normally adequate growth medium inhibited the growth of *Lactobacillus leichmannii* 313,[3] and that this inhibition could be reversed with either vitamin B_{12} or thymidine. The amount of vitamin B_{12} required for half maximal growth was found to be a function of the ionic strength (μ) of the growth medium, and it could be expressed by the equation

$$\log [B_{12}]_{1/2\max.} = a + b\mu \qquad (1)$$

In successive experiments, to be presented in this paper, we were able to extend the originally narrow "reversible range," *i.e.*, the limits of ionic strength between which the above relationship can be demonstrated. This was made possible by a slight modification of the experimental conditions. As a consequence, sufficiently distant points could be obtained to allow calculation of the numerical values of the constants a and b with a reasonable degree of accuracy.

In addition, the reversal of the salt inhibition with thymidine could be studied more extensively, as this material became commercially available. Furthermore, the influence of the salt concentrations on the "uptake" of vitamin B_{12} by the bacterial cells was investigated, and the results of these experiments are presented in this paper.

The hypothesis of "ionic inhibition" suggested in the preliminary publication is discussed in more detail in the light of the new experiments and in connection with the calculated values of the equation constants.

Experimental Part

The *Lactobacillus leichmannii* 313, strain ATCC 7830, culture was carried by weekly stab transfers in stock medium containing 0.75% yeast extract, 0.75% peptone, 1.0% dextrose, 0.20% KH_2PO_4, 10% (by volume) filtered tomato juice, 0.10% Tween 80, and 1.5% agar, *p*H 6.8. The daily inoculum was prepared by the addition of a loopful of the organism from the stab culture to the broth medium as above, and incubated at 37° for 24 hours. This culture was then centrifuged and washed three times with sterile saline. One drop of a 1:50 dilution was added to each tube containing 5 ml. of medium which included the salt or reversing agent, or both. After 20 hours incubation at 37° the per cent. transmissions were measured on a Coleman Universal Spectrophotometer Model 11A, at 660 mμ.

Basal stock solutions were prepared and kept in the refrigerator. All solutions and media for assay studies were prepared with twice distilled water. The medium was prepared from the stock solutions by combining them in the following order (per 100 ml.): 60 ml. of twice distilled water; 20 mg. of D,L-α-alanine; 10 mg. of L-cysteine hydrochloride; 1 ml. of salts A[4]; 1 ml. of salts B[5]; 100 mg. of Tween 80; 10 mg. of asparagine; 20 mg. of D,L-tryptophan; 1 mg. of adenine sulfate; 1 mg. of guanine hydrochloride; 1 mg. of uracil; 1 mg. of xanthine; 100 μg. of thiamine hydrochloride; 200 μg. of pyridoxine; 60 μg. of pyridoxamine; 60 μg. of pyridoxal; 100 μg. of calcium pantothenate; 200 μg. of niacin; 20 μg. of PABA; 0.2 μg. of biotin; 100 μg. of riboflavin; 0.40 μg. of folic acid; and 2.0 mμg. of vitamin B_{12}. The dry constituents were then added as follows: 2.0 g. of dextrose; 0.5 g. of anhydrous sodium acetate; 0.2 g. of ascorbic acid; and 0.5 g. of acid hydrolyzed casein (Difco). The *p*H was adjusted to 6.0 with a 5 *N* sodium hydroxide solution, and water was added to make 80 ml. of a 1.25-fold medium. Four ml. of this 1.25-fold medium was added to matched Coleman rimless test-tubes, which contained 1.0 ml. of the salt solutions or 1.0 ml. of the reversing agent solutions. These tubes were capped and autoclaved for 8 minutes at 126°. The values were expressed per 5-ml. tube.

For the salt inhibition experiments, the salt solutions were prepared at five times the required final concentration, and 1.0 ml. of these solutions was added to 4.0 ml. of the 1.25-fold medium. For the reversal studies, the reversing agents were diluted and 1.0 ml. of each dilution was added to 4 ml. of the 1.25-fold medium. In this case, the medium had been supplemented with 1.25 times the amount of salt needed in the respective experiment.

Recovery of vitamin B_{12} activity from salt inhibited cells and medium was accomplished by centrifugation of the assay tubes after they had been incubated for 16 hours and after their turbidity readings were recorded. The cells were then suspended in distilled water and autoclaved for 8 minutes. The autoclaved cells were centrifuged again and aliquots of the cell fraction extract, and of the medium fraction, were assayed for vitamin B_{12} activity in a normal assay medium without added vitamin B_{12}.

Results

Constants a and b of Equation 1.—Table I shows the turbidometer readings recorded in a typical "ionic inhibition" experiment. From these values, the vitamin B_{12} growth curve was plotted, for each of the salt concentrations. The vitamin B_{12} levels corresponding to "half maximal" growth (*i.e.*, to a turbidometer reading of "55") were determined from these curves, and, in Fig. 1, the logarithms of these values are plotted *versus* the ionic strength of the salt solutions.

(1) A preliminary report was presented before the Division of Biological Chemistry of the American Chemical Society, 124th National Meeting, September, 1953, Chicago, Illinois.

(2) T. J. Bardos and H. L. Gordon, THIS JOURNAL, **75**, 2019 (1953).

(3) Other investigators independently noted various manifestations of this salt effect. J. J. Corbett (*J. Bact.*, **64**, 889 (1952)) reported on the effect of a certain concentration (15 mg. per ml.) of NaCl on the dosage-response curve of *L. leichmannii* to vitamin B_{12}. D. Hendlin and J. Wall (*ibid.*, **67**, 38 (1954)) studied the effect of other inorganic salts, at various concentrations, and stated that their own results were in agreement with our published data and conclusions.

(4) Salts A: KH_2PO_4, 10%; K_2HPO_4, 10%.

(5) Salts B: $MgSO_4 \cdot 7H_2O$, 4%; $FeSO_4 \cdot 7H_2O$, 0.2%; $MnSO_4 \cdot 4H_2O$, 0.4%; NaCl, 0.2%.

TABLE I

TURBIDOMETER READINGS OBTAINED IN IONIC INHIBITION EXPERIMENT WITH SODIUM CHLORIDE

Vit. B_{12}[a]	NaCl, % 1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.86	2.02	2.18	2.34
0	100[b]	100	100	100	100	100	100	100	100	100	100
0.10	99	100	100	..	..	..	..	..	..	..	..
0.25	60	93	100	97	..	..	..	..	..	..	..
0.50	39	53	67	100	..	..	..	..	..	..	..
1.0	15	26	38	75	100	100	100	100	100	100	100
2.0	11	13	14	28	34	64	68	73	86	100	100
2.5	11	13	14	17	21	36	48	67	87	100	100
3.5	10	11	13	15	18	26	34	53	62	71	96
5.0	10	10	13	14	18	26	28	40	43	59	72
10	..	..	..	..	16	22	27	40	35	44	60

[a] mμg. per 5 ml., *in addition to* the 0.10 mμg. already present in the medium. [b] Turbidometer readings: % transmission; no growth = 100% transmission; maximal growth = 10% transmission; "half maximal" growth = 55% transmission.

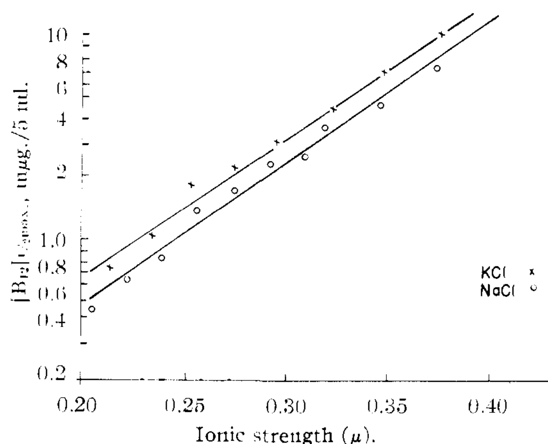

Fig. 1.—Vitamin B_{12} requirement for half-maximum growth of *L. leichmannii* as a function of the ionic strength. Abscissa: ionic strength of the medium due to the added salt. The ionic strength of the basal medium (approximately 0.14) is not included. Ionic strength $\mu = {}^1/_2\Sigma cv^2$ where c = gram ions per liter; v = valence, for each ion. In the case of NaCl and KCl, $\mu = M$, that is, the molar concentration. Ordinate: total vitamin B_{12} concentration (including the 0.10 mμg. per 5 ml. present in the medium) required for half-maximum growth (log scale).

It is apparent from Fig. 1 that for NaCl and KCl the straight line relationship exists through a longer range than was previously observed[2] in the 16 hours incubation experiments.

The regression equations represented by the straight lines in Fig. 1 were calculated by the method of least squares.[6]

These equations are

$$\text{for NaCl: } \log\ [B_{12}]_{1/2\text{max.}} = -1.75 + 7.05\ \mu_{\text{NaCl}} \quad (2)$$

$$\text{for KCl: } \log\ [B_{12}]_{1/2\text{max.}} = -1.56 + 6.84\ \mu_{\text{KCl}} \quad (3)$$

If we extrapolate equations 2 and 3 to $\mu_{\text{NaCl}} = 0$, and $\mu_{\text{KCl}} = 0$, respectively, then $\log\ [B_{12}]_{1/2\text{max.}}$ will be equal to the respective intercepts (*i.e.*, constant a of equation 1).

Consequently, the vitamin B_{12} concentration required for half maximal growth *in the absence of the salts* must be equal to the antilogarithms of the intercepts; the values so obtained are 0.018 mμg. per 5 ml. from the NaCl equation and 0.027 mμg. per 5 ml. from the KCl equation. The actual experimental value, determined from the "standard" B_{12}-growth curve, is about 0.020 mμg. per 5 ml., which is in agreement with the values calculated from the NaCl and KCl equations.

The "slope constants" (or regression coefficients; constant b of equation 1) are tabulated, with their respective "standard errors," in Table IV, and their significance will be discussed.

Reversal with Thymidine.—Instead of vitamin B_{12}, thymidine can be used to maintain growth of *L. leichmannii*[7-10] or to reverse the inhibition caused by the salts.[2] The concentration of thymidine required for half maximal growth increases with the ionic strength of the medium, but this increase is less pronounced than that of the vitamin B_{12} requirement. This is demonstrated in Table II for KCl. The figures in the last column show the decrease of the *relative* thymidine *vs.* vitamin B_{12} requirement with increasing ionic strength.

TABLE II

IONIC INHIBITION OF GROWTH IN PRESENCE OF THYMIDINE

KCl added to basal medium %	μmoles	$[\text{Thymidine}]_{1/2\text{max}}$ μg./tube	$\frac{[\text{Thymidine}]_{1/2\text{max}}}{[B_{12}]_{1/2\text{max}}}$[a]
0	0	0.67	50,000[a]
1.6	.214	0.90	1,250
1.9	.254	1.10	630
2.2	.295	1.50	520
2.4	.322	2.25	450
2.6	.348	3.00	350

[a] Expresses the amount of thymidine equivalent in growth effect to unit weight of vitamin B_{12}.

Recovery of Vitamin B_{12} Activity from Salt Inhibited Cells.—Experiments were carried out to test the possibility that "ionic inhibition" is due to an effect of the salts on the "uptake" of vitamin B_{12} by the cells. After 16 hours incubation period followed by turbidity readings, the cells were separated from the medium and autoclaved in distilled water. The "cell-extract" so obtained, as well as the medium were assayed for vitamin B_{12} activity. The results are shown in Table III. Approximately

(6) The authors are indebted to Miss Florence Pohley for calculating the regression equations.

(7) E. E. Snell, E. Kitay and W. S. McNutt, *J. Biol. Chem.*, **175**, 473 (1948).

(8) W. Shive, J. M. Ravel and R. E. Eakin, THIS JOURNAL, **70**, 2614 (1948).

(9) W. Shive, J. M. Ravel and W. M. Harding, *J. Biol. Chem.*, **176**, 991 (1948).

(10) E. Kitay, W. S. McNutt and E. E. Snell, *ibid.*, **177**, 993 (1949).

10% of the originally added vitamin B_{12} could be recovered from the cell-fractions in an active form by the arbitrary and crude method described above. However, even these amounts were more than sufficient for a full growth response in a normal medium. This fact is clearly demonstrated from the data of tubes No. 3 through 6 (Table III) in which no appreciable growth was obtained during the 16 hours incubation period (see column three). Centrifugation of these tubes gave barely visible residues, yet the vitamin B_{12} activities recovered from these centrifugation-residues (cell-fractions) were sufficient for complete growth in normal medium. At the same time, very little if any vitamin B_{12} activity could be recovered from the media of these salt-inhibited cultures.

TABLE III

RECOVERY OF VITAMIN B_{12} ACTIVITY FROM *L. Leichmannii* CELLS AND MEDIUM AFTER 16 HOURS INCUBATION IN SALT CONTAINING MEDIA (2.4% KCl)

Tube no.	Vitamin B_{12}, mμg./tube	Galvanometer readings after 16 hr. incubation	*Assay of cell material* B_{12} activity recov. from cells after autoclaving for 8 min. with water, mμg./tube	*Assay of medium* B_{12} activity recovered from medium, mμg./0.5 cc.[b]
1	0.5	98[a]	0.035	0
2	1.0	100	.070	0
3	1.25	100	.125	0
4	1.5	100	.15	0
5	2.5	100	.27	.0075
6	3.0	83	.33	.015
7	3.5	74	.38	.025
8	4.0	67	.44	.04
9	5.0	49	.20	.09
10	10	22	.30	.20
11	25	12	1.00	.50
12	50	10	1.25	1.25

[a] % transmission; no growth at 100% transmission; maximum growth at 10% transmission. [b] Maximum error (in cell-assay) due to incomplete removal of medium.

These data indicate that the inhibition caused by the salts cannot be explained as merely due to an effect of these salt concentrations on the vitamin B_{12} "uptake," even though at high vitamin B_{12} levels (tubes No. 7–12) increasing amounts of the vitamin could be recovered from the medium.

Discussion

There are obviously several possible explanations for the phenomena which we termed[11] "ionic inhibition." One such explanation could be based on the discovery of Davis and Chow,[12] that sodium chloride inhibits the "uptake" of vitamin B_{12} by resting bacterial cells. However, the salt concentrations used in our experiments are too low in comparison to the salt concentrations which were reported to cause a significant inhibition of "uptake." In addition, our vitamin B_{12} recovery experiments reported in this paper, seem to eliminate such an effect of the salts as a satisfactory explanation for the growth inhibition.

As we indicated in our preliminary publication,[2] it is possible to arrive theoretically to a functional relationship between $[B_{12}]_{1/2\max}$ and the ionic strength which is similar in form to the experimentally obtained equation 1. If we assume that vitamin B_{12} reversibly combines with an "apoenzyme," E_a, to give the actual "growth limiting" factor, E_aB_{12}

$$B_{12} + E_a \rightleftharpoons E_aB_{12} \quad (4)$$

then the equilibrium constant of this reaction

$$K = \frac{[E_aB_{12}]}{[B_{12}][E_a]} \quad (5)$$

can be written in the form

$$\log K = \log [E_aB_{12}] - \log [B_{12}] - \log [E_a] \quad (6)$$

If we further assume that the available concentration of E_a is proportional to its solubility, *i.e.*

$$[E_a] = \alpha S \quad (7)$$

which in turn is a function of the ionic strength, assuming that Cohn's "salting-out" equation for proteins applies[13]

$$\log S = \beta - K_s\mu \quad (8)$$

where β and K_s are Cohn's salting out constants, then

$$\log [E_a] = \log \alpha + \beta - K_s\mu \quad (9)$$

Substituting for $\log [E_a]$ in (6)

$$\log K = \log [E_aB_{12}] - \log [B_{12}] - \beta' + K_s\mu \quad (10)$$

where the new constant $\beta' = \beta + \log \alpha$. Furthermore, if E_aB_{12} is the growth limiting enzyme, then the growth rate, r, is proportional to the concentration of E_aB_{12}

$$r = k[E_aB_{12}] \quad (11)$$

and for constant growth rate (*e.g.*, half maximal growth per 20 hr.)

$$[E_aB_{12}]_{1/2\max} = C \text{ (constant)} \quad (12)$$

Substituted into (10)

$$\{\log K = C - \log [B_{12}] - \beta' + K_s\mu\}_{1/2\max} \quad (13)$$

or

$$\log [B_{12}]_{1/2\max} = C - \log K - \beta' + K_s\mu \quad (14)$$

and combining the constants

$$\log [B_{12}]_{1/2\max} = \text{const.} + K_s\mu \quad (15)$$

By comparing equations 15 and 1, it follows that

$$b = K_s \quad (16)$$

That is, if the above assumptions are true, then the "slope constant" of our empirical equation 1 must be identical with the "salting-out" constant K_s of the hypothetical apoenzyme, E_a. Since the K_s constant of Cohn's equation is "a characteristic of the protein,"[13] a comparison of the values obtained for the slope constant b with the reported K_s values of various proteins should give some indication regarding the possible nature of E_a.

Table IV summarizes the slope constants calculated from the data obtained in two different ionic inhibition experiments, as well as the ionic strength range in which these experiments were carried out. It is apparent that the slope constant b is much larger than the K_s values reported by Cohn for various proteins,[13] which are of the order of 0.5–1.5. In addition, the area of ionic strength at which most proteins are "salted-out" from their solutions is

(11) To express its dependence on the ionic strength.

(12) R. L. Davis and B. F. Chow, *Science*, **115**, 351 (1952).

(13) E. J. Cohn, *Ann. Rev. Biochem.*, **4**, 133 (1935).

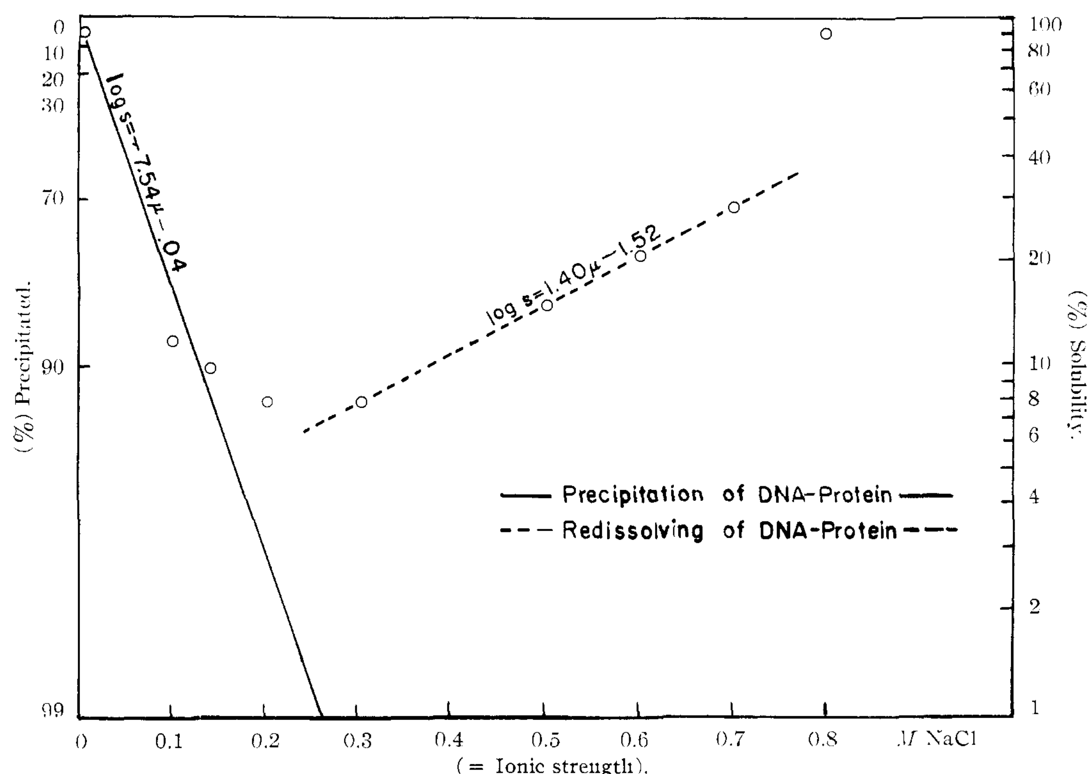

Fig. 2.—Solubility of DNA protein as a function of the ionic strength.

about ten times higher than the "reversible range" of ionic inhibition.

However, there is one known exception: the *desoxyribonucleoprotein* obtained from sea-urchin eggs[14,15] and other sources[16] is reported to be precipitated by 0.14 molar solution of NaCl and redissolved at higher salt concentrations into a dissociated, irreversibly changed form. Assuming that this latter form is enzymatically inactive, we attempted to estimate the per cent. of DNA-protein that remained in solution without being first precipitated and then redissolved. If we separate the NaCl-solubility diagram of Bernstein and Mazia[15] into two components, the left hand, ascending part of the curve corresponds to the precipitation ("salting-out") while the right hand, descending part corresponds to the redissolving of the nucleoprotein. We extrapolated the former beyond the 0.14 M point in order to obtain a theoretical salting-out curve that asymptotically approaches the "100%" ordinate. If the points of this curve are plotted on a logarithmic scale (Fig. 2) they can be approximated by a straight line which, when extrapolated, would intersect the "99%" ordinate (=1% solubility") at 0.26 M, the "99.9%" ordinate (= 0.1% solubility) at 0.4 M and the "99.99%" ordinate (0.01% solubility) at 0.54 M concentration of NaCl. Thus, through the area of ionic strength corresponding to the "reversible range" of ionic inhibition, the per cent. of "unchanged" DNA-protein contained in solution decreases from about 0.5% to 0.01%. This is compatible with the postulated behavior of E_a. The K_s constant of DNA-protein, calculated from the ascending part of the NaCl solubility diagram[15] is 7.5, which is a distinctly higher value than that of other proteins[13] and is of the order of magnitude of the slope constant b (7.08 for NaCl, see Table IV).

(14) M. H. Bernstein and D. Mazia, *Biochim. Biophys. Acta*, **10**, 600 (1953).

(15) M. H. Bernstein and D. Mazia, *ibid.*, **11**, 59 (1953).

(16) A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.*, **30**, 117 (1946).

TABLE IV[6]

CALCULATED VALUES OF SLOPE, b, AND STANDARD ERROR, s_b

	b	s_b
NaCl	7.08	0.32
KCl	6.84	.26

Range of Observations

	Lower limit	Upper limit
Ionic strength of basal media	0.14	0.14
Ionic strength of added salts (μ)	.20	.40
Total ionic strength	.34	.54

These data suggest that "E_a" may be a desoxyribonucleoprotein. This conclusion, as well as our hypothesis of "ionic inhibition" itself, depends on the validity of a number of assumptions which were stated in the course of the preceding discussion. More direct evidence is obviously needed to prove the existence of a "DNA-protein-vitamin B_{12} enzyme"; however, the indirect evidence now obtained as the result of a quantitative analysis of ionic inhibition experiments, is the first suggestion of the existence of such an enzyme. The implications of this are intriguing in view of the apparent catalytic role of vitamin B_{12} at some stage of DNA-synthesis.[7,8,17] If "E_a," by definition a part of the

(17) L. D. Wright, H. R. Skeggs and J. W. Huff, *J. Biol. Chem.*, **175**, 475 (1948).

"B_{12} enzyme," is itself a DNA-protein, it could play the role of an *auto catalyst* in the biosynthesis of new DNA-protein. Such an autocatalytic mechanism would be, of course, in agreement with the observed exponential kinetics of this biosynthetic process.

Further work, now in progress, will test the validity of our hypothesis, which for the time being may be regarded as a tentative explanation of the experimental facts presented in this paper.

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CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

A Method for the Quantitative Determination of C-Terminal Amino Acid Residues[1]

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A carboxoid method which is analogous to a previously described aminoid method is reported. This C-terminal method has been evaluated on amino acids, dipeptides, tripeptides, lysozyme and in other work on lysozyme proteolyzates. The method appears to offer a number of advantages over other C-terminal procedures which have been described. Among these are stereospecificity, quantitative applicability, and ease of operation such as freedom from the need for fractionation. Principal discernible limitations are behavior which is less quantitative with dipeptides than with larger peptides and incomplete reactivity of terminal aspartic acid, glutamic acid and proline residues. When applied to lysozyme, one C-terminal residue of L-leucine was found. Of fourteen other types of residue assayed in the experiment designating C-L-leucine no other was significantly altered by the treatment with ammonium thiocyanate and acetic anhydride.

A stepwise N-terminal method, representing a modification of the original Abderhalden[3] and Edman[4] methods has been applied with quantitative results to synthetic peptides[5] and to subtractive structural studies of peptides and mixtures of peptides.[6,7] These modifications have been useful also in qualitative structural studies.[8,9] The utility of such a method would be greatly extended if it became possible to determine what proportions and types of C-termini are free, or the proportions and types of carboxoid residue liberated simultaneously with the aminoid, particularly in fragmentation studies.

A number of methods of assignment of C-termini has been reported.[10—21]

It should be stated parenthetically that, aside from chemical methods, carboxypeptidase has been employed already for C-terminal studies.[22,23,16] Uncertainties[10,24,25] in the application of proteases to prior fragmentation for structural studies, which might however be resolved by use of model synthetic peptides, yet cloud the picture. A study of proteolysis made possible in part by the carboxoid method described in this paper indicates, in fact, that concordance of sequential assignments, after fragmentation with different proteases, does not assure freedom from transpeptidation.[26] This follows inasmuch as results show that the substrate(s) is itself a primary determinant of the nature of the reaction.[26,27] Difficulties of an incompletely defined sort have been recorded for the carboxypeptidase technique.[28] However the discrepancies with enzymes are resolved,[21] the need for a quantitative carboxoid method is further emphasized.

The type of method which appeared to offer the most promise for quantitative and stepwise adaptations was that developed in its original qualitative form by Schlack and Kumpf[29] from the Johnson reaction[30,31] and applied extractively to proteins in later work.[15,18,19,21] This method was selected also because of the likelihood that some of the accumulated information on the similar phenyl isocyanate[10] and phenyl isothiocyanate methods

(1) Journal Paper No. J-2678 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project No. 863. Supported by Grant G-3025 (C) from the National Institutes of Health, U. S. Public Health Service. Largely from the Ph.D. thesis, in Food Technology, of Thomas L. Hurst (1953).

(2) Du Pont Fellow, 1953-1954.

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(23) J. I. Harris and C. H. Li, *ibid.*, **74**, 2945 (1952).

(24) A. K. Balls and E. F. Jansen, *Ann. Rev. Biochem.*, **21**, 20 (1952).

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