CORRELATION OF THE N $\rightleftharpoons$ a REACTION OF THYROGLOBULIN WITH THE TYPE OF BREAKDOWN PRODUCED BY PAPAIN*

IAN J. O'DONNELL**, ROBERT L. BALDWIN AND J. W. WILLIAMS

Departments of Chemistry, Biochemistry, and Dairy and Food Industries, University of Wisconsin, Madison, Wisc. (U.S.A.)

Study of the initial stages in which a protein is broken down by a proteolytic enzyme can yield information about the following questions. (1) Do intermediates accumulate in the digestion mixture? (2) Is there a single pathway of breakdown for all of the protein molecules? (3) How do the properties of various intermediates compare with those of the starting material? These are related problems and information about any of them provides some insight into the others. We shall be concerned here with the first of these.

Whether or not intermediates are found during the digestion is, as LINDERSTRØM-LANG[1,2] has shown, a question of reaction rates: that is, are the intermediates broken down much more rapidly than the starting material, or at comparable rates? In the first instance only native protein and end products are observed during all stages of the digestion. This is called a *one-by-one breakdown*; many examples are known[3–8]. The type of breakdown in which intermediates are degraded at rates comparable to that of the disappearance of native protein will be called here *interrupted breakdown*. Several examples of this also are known[4,9–13]. In the digestion of horse diphtheria antitoxin, fragments with increased specific antibody activity can be obtained[9]. Also several systems have been studied in which there is a major, if not unique, pathway of breakdown. Thus specific intermediates have been obtained from the limited digestion of ovalbumin by subtilisin[14–16], from the peptic digestion of ribonuclease[17], and from the tryptic breakdown of myosin[18].

In the one-by-one breakdown there is a stage in the degradation of each molecule in which the protein becomes labile to attack by enzyme and then is broken down rapidly. This labile form may arise in one of two ways[1,19,20]: it may be in equilibrium with the native form or it may be produced by enzymic attack upon the native form. Examples of enzymic degradation in which there is an equilibrium between native and labile forms of the protein are the autodigestion of trypsin[21,22] and the tryptic breakdown of β-lactoglobulin[1,20]. In order to find out why the intermediates are labile, one would like to compare chemical and physical properties of the native and labile forms. A labile intermediate produced by enzymic action cannot in general be isolated; if it could, it would not be a labile intermediate. However when a labile

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** Permanent Address: Wool Textile Research Laboratories C.S.I.R.O., Melbourne, Australia.

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form is in equilibrium with the native form, one may study the properties of either form by finding conditions where the equilibrium favors that form.

Hog thyroglobin at low ionic strength undergoes a reversible reaction which apparently is a change in configuration. LUNDGREN AND WILLIAMS[23], who have described this reaction, call it the N ⇌ α reaction. The purpose of this work was to study the enzymic breakdown of the N and α forms, to see if they showed any interesting differences and, if so, to find whether these could be related to properties of the N and α forms. Since thyroglobulin has a molecular weight of 660,000, the ultracentrifuge can resolve the larger fragments produced by degradation and so sedimentation analysis was used. Papain was chosen as a proteolytic enzyme because its action can be stopped readily with iodoacetamide.

EXPERIMENTAL

Protein preparation

The thyroglobulin was prepared from fresh hog thyroids by the method of DERRIEN, MICHEL AND ROCHE[24] in which a saline extract of the thyroid glands is fractionally precipitated with ammonium sulphate. The thyroglobulin thus obtained was thoroughly dialyzed against dilute potassium chloride and then against distilled water. Most of the preparation was stored in the frozen state at a protein concentration of about 4 g/100 ml; approximately 1 g was lyophilized.

The crystalline bovine plasma albumin was lot No. 212,113 from Armour and Co.; the crystalline papain was obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

Protein concentration

The concentration c, expressed in g/100 ml of solution, was determined by the Kjeldahl nitrogen procedure, 15.8% being taken as the nitrogen content of thyroglobulin[25]. The quantity $\Delta n/\Delta c$, which approximates the differential refractive increment dn/dc, was found to be $1.84 \cdot 10^{-3}$ for thyroglobulin in the cacodylate buffer (below) and in the neighborhood of $c = 1$.

Buffers

The buffer solutions had the following compositions and properties.

pH 6.40: 0.1500 M KCl, 0.0073 M cacodylic acid, 0.0200 M potassium cacodylate; viscosity relative to water at 25° = 1.010_7, density at 25° = 1.0066_8 g/ml.

pH 6.65: 0.15 M NaCl, a mixture of Na_2HPO_4 and NaH_2PO_4 with a total phosphate molarity of 0.025; viscosity relative to water at 25° = 1.026_8, density at 25° = 1.0058_4 g/ml.

pH 4.60: 0.1500 M KCl, 0.0100 M acetic acid, 0.0100 M potassium acetate; values for the density and relative viscosity of this buffer were taken from previous work[26].

All components were of reagent quality with the exception of the cacodylic acid. For the buffers at pH 6.40 and at pH 4.60 all salts were dried overnight in vacuum, at approximately 60°, and the solutions were made up by weighing each component, with the weights corrected to readings in vacuum. The pH measurements were made with a glass electrode at room temperature (25° ± 3° C).

Papain digestion of thyroglobulin

All digestions were carried out at 39.5°. In one series, designated "in salt", the reaction mixture contained papain, a solution of 1% (w/v) thyroglobulin in the cacodylate buffer described above, and also $5 \cdot 10^{-3}$ M cysteine and $1 \cdot 10^{-3}$ M versene (the tetrasodium salt of ethylenediamine tetraacetic acid) for activation of the papain[27]. Samples were removed at various times during the digestion and the action of the papain was stopped by making the sample 0.01 M in iodoacetamide. In another series, termed "very low salt", the digestion mixture contained, in addition to papain, 1 g/100 ml thyroglobulin, $5 \cdot 10^{-4}$ M cysteine, $5 \cdot 10^{-4}$ M KCl, $1 \cdot 10^{-4}$ M versene, $5 \cdot 10^{-4}$ M potassium cacodylate and $1.85 \cdot 10^{-4}$ M cacodylic acid. In addition to the iodoacetamide used to stop the reaction, concentrated cacodylate buffer was added of the same relative composition as the pH 6.40 buffer described above so that the arrested samples were 0.002 M in iodoacetamide and 0.15 M in KCl, in addition to the cacodylate and the components of the reaction mixture. The concentration of papain in the digestion mixture was either 0.01 or 0.0025 g/100 ml. Control experiments showed that the iodoacetamide arrested the digestion effectively.

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For experiments in which dioxane was present, these digestion mixtures were made 10% (v/v) with respect to dioxane. The dioxane was a preparation containing only 19 p.p.m. of benzene*.

Diffusion

A Gouy diffusiometer which has been described previously[28,29] was used with a 9 cm Tiselius-type cell. At the beginning of each experiment the boundary was sharpened by the capillary method. The protein solutions were dialyzed in cellophane tubing against two changes of buffer, for at least 48 h at 4°, before coming to final equilibrium at 25° by standing overnight in the diffusion bath. The diffusion experiments were performed within 0.015° of 25°, and the temperature during an experiment was constant to 0.002°. Measured values of the reduced height area ratio were corrected to 25° by use of the Stokes-Einstein relation, and to the viscosity of water by multiplying by the relative viscosity of the buffer.

The reduced height area ratio[30] is given by

$$\mathcal{D}_A = \frac{(\Delta n)^2}{4\pi t\,(\delta n/\delta x)^2_{\max}} \tag{1}$$

where Δn is the difference in refractive index across the boundary, t is the time from the beginning of diffusion, x is position in the cell and $(\delta n/\delta x)_{\max}$ is the maximum refractive index gradient. Both $\mathcal{D}_A$ and the reduced fringe-deviation graphs, the latter of which show the departure of the refractive-index gradient curve from Gaussian form, were evaluated from equations 35 and 38 of DUNLOP AND GOSTING[30]. In order to take account of the imperfectly sharp initial boundaries, the reduced height area ratios were extrapolated to infinite time by the method of LONGSWORTH[31].

Viscosities

Viscosities were measured by use of an Ubbelohde viscometer which has a flow time of 265.8 sec with water at 25°. The bath was thermostated within 0.005° of 25.00° C. Viscosities were computed from the following equation, which includes a correction for the kinetic energy effect.

$$\eta = \varrho\,(At - B/t) \tag{2}$$

In equation (2), η is the viscosity, ϱ is the density, t is the flow time and A and B are constants for the viscometer.

Sedimentation

Sedimentation experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with temperature control. The experiments were carried out within 0.2° of 25° and the temperature of each experiment was constant within 0.04°. Sedimentation coefficients were corrected to water at 25° by the relation

$$s\eta/(1 - \bar{v}\varrho) = \text{constant} \tag{3}$$

where s is the sedimentation coefficient at a given concentration, and $\bar{v}$ is the partial specific volume. A value of 0.723 ml/g was used for $\bar{v}$[32] of thyroglobulin. The sedimentation coefficients were computed by a method[33] which takes account of the dependence of s on concentration. From a series of experiments at different concentrations, values of an apparent sedimentation coefficient s^* are taken at fixed values of $s_0\omega^2(t + t_0)$, and the plot of these values of s^* against the initial concentration c_0 gives s_0, the sedimentation coefficient at infinite dilution, and also data from which the true dependence of s on concentration can be calculated readily.

$$s^* = \frac{\ln(r_b/r_b^0)}{\omega^2(t - t_0)} \tag{4}$$

Here ω is the angular velocity, r_b is distance from the position of the boundary to the center of rotation, r_b^0 is the value of r_b at the time of the first photograph, t is the effective time of sedimentation (*i.e.* t is corrected for the smaller values of ω during the period of acceleration), and $(t - t_0)$ is the time from the first photograph.

The variation of s with c was determined for native thyroglobulin in the cacodylate buffer of pH 6.40. The solutions were made by diluting a stock solution of protein in buffer with weighed amounts of buffer. Knowing the protein concentration and density of the stock solution, and the density of the buffer, one is able to compute the concentration of protein in the diluted solution on the assumption that changes of volume on mixing are negligible.

Approximate distributions of sedimentation coefficient were obtained by extrapolation to

* The dioxane was kindly given to us by Dr. M. A. STAHMANN.

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infinite time of an "apparent distribution"[34-36]. A reduced coordinate s, with the units of sedimentation coefficient, is defined by

$$s \equiv [\ln (r/r_0)]/\omega^2 t \tag{5}$$

where r is distance from the center of rotation and r_0 is the position of the meniscus. The apparent distribution of sedimentation coefficient is defined by

$$g^*(s) \equiv \frac{\frac{\delta n_c}{\delta r}\omega^2 t r^3}{n_c^0 r_0^2} \tag{6}$$

where $\delta n_c/\delta r$ is that part of the refractive index gradient produced by sedimentation of the protein and n^0 is the difference in refractive index between the initial protein solution and the buffer. If there is no dependence on concentration of the sedimentation coefficients, and if the ratio of boundary spreading caused by heterogeneity to that caused by diffusion is in the proper range, then extrapolation of $g^*(s)$ to infinite time yields the true distribution of sedimentation coefficients, $g(s)$. The distributions shown here are denoted by $g(s)$, to indicate that the effects of diffusion have been removed (or in some instances diminished) by extrapolation to infinite time, but that no correction has been made for the dependence of sedimentation coefficients on concentration. In extrapolating $g^*(s)$ to infinite time, the variable used for extrapolation was $(1/t)$.

The integral distribution $G(s)$ is defined by

$$G(s) \equiv \int_0^s g(s)\, ds \tag{7}$$

In Fig. 7 the integral distribution curve was computed from the corresponding values of $g(s)$ by trapezoidal integration and the value of $G(s)$ at $s = 0$ was found from the condition that $G(s) = 1$ for $s = \infty$.

The photographic plates were measured by means of a coordinate comparator with two-way motion. The plates were aligned so that the meniscus trace was parallel to one axis of motion. A double sector cell was used in most of these experiments; this has the advantage of direct recording of the baseline. A phase-plate with a metal-line deposit at the phase boundary was used as a schlieren diaphragm, and also a wire was used in some of the earlier experiments.

RESULTS

Characterization of native thyroglobulin by sedimentation and diffusion

Diffusion. The results of the diffusion experiments are given in Table I. For a two-component system in which there are no volume changes on mixing and in which the diffusion coefficient is independent of concentration, the reduced height-area ratio $\mathcal{D}_A$ is equal to D, the diffusion coefficient of the system and the deviation graph is everywhere zero. For three-component systems (*i.e.* two solutes and a solvent) subject to the same qualifications, $\mathcal{D}_A$ is equal to the diffusion coefficient of the solute

TABLE I

RESULTS FROM DIFFUSION EXPERIMENTS WITH THYROGLOBULIN AND BOVINE ALBUMIN

Protein	$10^3 \times \Delta n$	*pH*	$10^7 \times (\mathcal{D}_A)^{(a)}_{25,\,buffer}$	$10^7 \times (\mathcal{D}_A)^{(a)}_{25,w}$	$10^4 \times \Omega_{max.}$
Thyrogl. (1)	0.692	6.40	2.81	2.84	69
Thyrogl. (2)	1.319	6.40	2.96	2.99	65
Thyrogl. (3)	1.753	6.40	2.82	2.85	61
Thyrogl. (4)	0.674	6.65	2.85	2.93	66
B.P.A.	1.946	4.60	6.50	6.52	22
B.P.A.	1.962	4.60	6.50	6.52	16
B.P.A.	1.800	6.40	6.79	6.86	13

(a) Units are $cm^2\ sec^{-1}$.

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present in different concentrations on the two sides of the boundary and the deviation graph is again everywhere zero, provided that there is no difference in concentration of the other solute across the boundary and provided that there is no interaction of the solute flows.

The reduced height–area ratios measured for thyroglobulin do not vary in a regular manner with Δn, and hence with concentration. Since $\mathcal{D}_A$ can be measured with a precision of 0.1% with this apparatus, it appears that the thyroglobulin solutions, either when stored in the frozen state or upon dialysis, undergo changes large enough to affect the diffusion measurements but not the sedimentation coefficient of the main component. When diffusion measurements made on the same preparation at different times are poorly reproducible, one cannot expect close agreement between diffusion measurements made with different preparations. However a reported value[32] of $2.60 \cdot 10^{-7}$ cm^2 sec^{-1} for the diffusion coefficient of thyroglobulin at 20° becomes $2.93 \cdot 10^{-7}$ when converted to 25°, and is in the range of values given in Table I.

Native thyroglobulin can be studied only above its isoelectric pH because it rapidly becomes denatured below the isoelectric point. Values of Ω, the reduced fringe deviation, were unusually large in the experiments with thyroglobulin (Fig. 1), showing that diffusing boundaries differed markedly from Gaussian shape. These values of Ω could be caused by heterogeneity[26] or by either of two factors which are related to performing the experiments away from the isoelectric pH. There could be concentration gradients of the buffer constituents across the boundary, resulting from the Donnan equilibrium, and there could be interaction of flows resulting in particular from electrostatic coupling of the ion flows[30]. Consequently a few diffusion experiments were performed with bovine albumin at its isoelectric point and at a pH 1.8 units higher. The values of Ω_{max} (Table I) at the two pH's were not significantly different. Because of this and because the sedimentation patterns of thyroglobulin show the presence of both slower and faster sedimenting components, it is likely that heterogeneity is major factor in producing the large deviation graphs of the diffusion experiments.

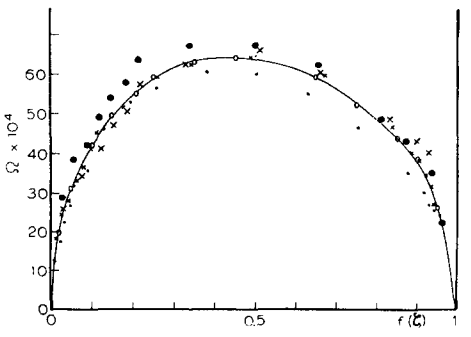

Fig. 1. Reduced fringe-deviation graphs from the diffusion experiments with thyroglobulin. These graphs show the deviation of the boundary from Gaussian form. Expt. 1 = ●, expt. 2 = ×, expt. 3 = •, expt. 4 = ×, average of all four experiments = ○. (Numbers refer to the experiments in Table I.)

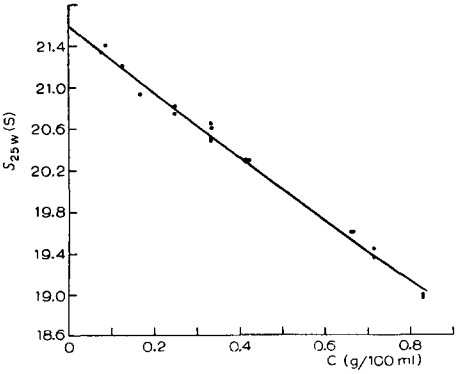

Fig. 2. The sedimentation coefficient of the main component of thyroglobulin as a function of concentration. The values of s are referred to water at 25° and have been computed by an equation which takes account of the variation of s with time caused by the progressive dilution with time in each experiment. The radius of each filled circle is 0.02 S.

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Two years ago a value of $(\mathcal{D}_A)_{25,w}$ was measured for this same sample of bovine albumin at pH 4.60 and found to be $6.66_1 \cdot 10^{-7}$ cm^2 sec^{-1} [37]. The discrepancy between this and the value given here indicates that bovine albumin can undergo changes on storage, even when stored in the lyophilized state at −5°. Light-scattering measurements[38] made with B.P.A. also have shown that aggregation can occur on storage.

Sedimentation. The sedimentation patterns revealed small amounts of components with sedimentation coefficients both less than and greater than that of the major component. Moreover the amount of the slower component changed with time of storage. (This is discussed in more detail later.) With the initial intention of testing the homogeneity of the major component by FUJITA's equation[39], we measured the dependence of its sedimentation coefficient on the total concentration. In order to use FUJITA's equation, one requires accurate knowledge of the dependence of s on c[33].

Twenty experiments performed in the concentration range 0.08–0.8 g/100 ml yielded sedimentation coefficients whose dependence on concentration could be represented by the quadratic equation

$$s_{25,w} = 21.59\ (1 - 0.147\ c + 0.006\ c^2)\ S \qquad (8)$$

with an average deviation of 0.22% (see Fig. 2). These experiments confirm the conclusion reached in work with bovine albumin[33] that the sedimentation coefficient of a well-behaved protein can be reproduced with a precision of 0.2% provided that one has accurate temperature control and access to a comparator, and provided that one makes use of a suitable method of computing s.

Equation 8 may be compared with the following values of s in the literature: an average ($0 < c < 0.4$ g/100 ml) value of $s_{20,w} = 19.2$ S[25] and extrapolated ($c = 0$) values of 19.4[32] and 18.7[40] S. When multiplied by the ratio of the viscosity of water at 20° to that at 25°, these values become 21.6, 21.9 and 21.1 S, respectively.

Use of Svedberg's equation for the molecular weight, together with the figures $s_{25,w} = 21.6 \cdot 10^{-13}$ sec, $D_{25,w} = 2.9 \cdot 10^{-7}$ cm^2 sec^{-1}, and $\bar{v} = 0.723$ ml/g[32], gives a value for the molecular weight of $6.6 \cdot 10^5$ g/mole. Because of the complexity of the system, sedimentation and diffusion measurements can be made far more precisely than the molecular weight can be determined.

The presence of slower and faster sedimenting components interfered with the test of homogeneity by FUJITA's equation[39], which is strictly applicable only to a single sedimenting solute.

The N ⇌ a reaction of thyroglobulin

Character of the reaction. This article contains little new information about the nature of the N ⇌ a reaction. We have confirmed the results of LUNDGREN AND WILLIAMS[23] that the a form can be observed by means of the ultracentrifuge in solutions of low ionic strength and that the N ⇌ a reaction is reversed by adding salt (Fig. 3).

Since the sedimentation coefficient of a is roughly half that of N, either the molecular weight of a must be less than that of N or the translational frictional coefficient of a must be greater. LUNDGREN AND WILLIAMS[23] found that the ratio of N to a decreases with increasing concentration; if the a form were a dissociation product, the reverse would have to be true. Thus the N ⇌ a reaction appears to be

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an isomerization reaction. The results of LUNDGREN AND WILLIAMS were based on areas under the peaks of the sedimentation patterns and correction for the JOHNSTON-OGSTON[41] effect would decrease the dependence of the N/a ratio on concentration. For an isomerization reaction one would expect this ratio to be essentially independent of concentration.

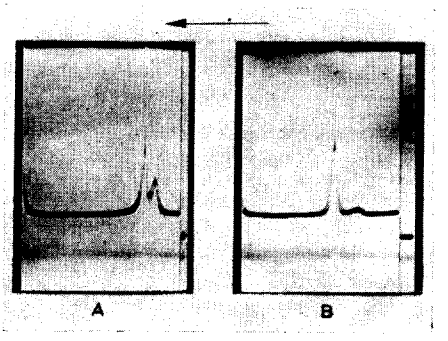

Fig. 3. Reversibility of the N $\rightleftharpoons$ a reaction of thyroglobulin. (A) Thyroglobulin dialyzed against distilled water. The faster-sedimenting component is native thyroglobulin and the slower-sedimenting one is the a form. (B) After making the solution 0.2 M in KCl. (The small amount of slower-sedimenting material appears to be irreversibly denatured protein, present under all conditions.) These schlieren patterns were recorded after approximately 20 min sedimentation at 56, 100 r.p.m. The protein concentration was 0.8 g/100 ml and the angle of the schlieren diaphragm was 65°.

Because the N and a forms show separate sedimenting boundaries, the rates of reaction must be slow: one can infer that the half-times are greater than the length of the sedimentation experiment[42]. Only the sedimentation studies afford certain evidence for existence of the a form. Any measurement of an average property, such as reduced viscosity or specific optical rotation, cannot distinguish between a mixture of two forms whose proportions depend on ionic strength and a single form whose properties depend on ionic strength. In this respect it is fortunate that the reaction rates are slow; otherwise the sedimentation measurements would fail to demonstrate the existence of the a form.

The conversion of N to a is not complete even in distilled water, at pH's where papain is reasonably active. Consequently we tried to shift the equilibrium towards a by means of various agents.

Effects of acid, urea and storage. Exposure to a pH below the isoelectric point of 4.6 results in irreversible denaturation and there is a shift in isoelectric pH to 5.0[25]. If the denatured form were fairly homogeneous in sedimentation behavior, it would be interesting to study its breakdown by papain. However the denatured protein shows heterogeneity in sedimentation behavior and undergoes aggregation.

Since the a form is observed only at low ionic strengths, it appears to be an expanded form produced by internal electrostatic repulsion and consequent breakage of weak, non-covalent bonds of the N form. If these bonds were hydrogen bonds one might expect urea to shift the N $\rightleftharpoons$ a equilibrium towards a. And fairly low concentrations of urea do have striking effects on the sedimentation patterns; however these effects are not reversible. Instead it was found that urea readily causes irreversible denaturation when the a form is present. An experiment was performed in which two samples of thyroglobulin were exposed to 1.7 M urea, one in distilled water and the other in 0.15 M KCl; the final pH's after adding urea were 6.7 and 6.6, respectively. Both samples were examined in the ultracentrifuge after dialyzing out the urea and adding an 0.01 M veronal buffer of pH 8.8. The sample which initially was salt-free and contained some of the a form showed more denaturation than the other.

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Examination of our thyroglobulin preparation soon after storage either in the lyophilized state or in frozen solution showed small amounts of both leading and trailing components. As time passed, the amount of slower-moving material increased in the sample stored in frozen solution. In Fig. 3 it appears as a small peak; in Fig. 4, which shows sedimentation experiments performed several months later, this peak is larger. (Other experiments showed this to be true whether or not dioxane was present.) Our information about this material is limited to the following facts: (a) it sediments more slowly than native thyroglobulin; (b) it increases upon storage in frozen, salt-free solution; and (c) it is degraded very rapidly by papain. We conclude tentatively that this material is irreversibly denatured.

Effects of dioxane. Since appearance of the α form is controlled by electrostatic forces, we investigated the effect of lowering the dielectric constant. Dioxane was chosen for this purpose because of its low dielectric constant and good miscibility with water.

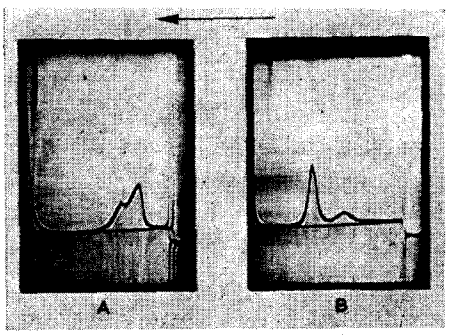

Fig. 4. Effect of 10% dioxane on the N ⇌ α reaction. (A) In distilled water plus 10% dioxane. (B) In cacodylate buffer plus 10% dioxane. These photographs were taken after approximately 70 min sedimentation at 39, 460 r.p.m. The protein concentration was 1 g/100 ml and the angle of the schlieren diaphragm was 65°.

Addition of 10% (v/v) dioxane to a solution of thyroglobulin in distilled water markedly changes the sedimentation pattern (Fig. 4) and the reaction is reversed by adding salt. We will still refer to this reaction in 10% dioxane as the N ⇌ α reaction because of its dependence on ionic strength and its reversibility. Fig. 4 shows that the α peak is now the major one and that the two peaks are not resolved. These facts suggest that the equilibrium has been shifted towards the α form and that the reaction rates have been increased.

In order to see if dioxane causes any irreversible effects, a solution of thyroglobulin was examined in the ultracentrifuge after the 10% dioxane had been removed by dialysis against cacodylate buffer. By qualitative inspection the sedimentation patterns were normal.

Properties of the α form. There are few physical properties which can be measured and interpreted reliably at the low ionic strengths at which the α form occurs. It is particularly difficult to measure molecular weights. In order to check the conclusion of LUNDGREN AND WILLIAMS[23] that the α form is not a dissociation product, we have measured the reduced viscosities, at constant concentration, of solutions containing different amounts of the α form. If the α form is a dissociation product, its viscosity

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increment might be less than that of the N form, whereas if it is an expanded form, its viscosity increment would certainly be greater than that of N. The results, which are given in Table II, show that the reduced viscosities are greater in solutions containing the α form. This is consistent with the concept of α being a swollen or unfolded form, but interpretation is complicated by the dependence of intrinsic viscosity on ionic strength (*cf.* TANFORD AND BUZZELL[43]).

It seems unlikely that the effect of dioxane on the N $\rightleftharpoons$ α reaction is only one of lowering the dielectric constant. Perhaps hydrophobic bonds are broken in the reaction from N to α.

TABLE II

REDUCED VISCOSITIES OF SOLUTIONS OF THYROGLOBULIN WITH VARYING AMOUNTS OF THE α-FORM

Solvent composition	*Concentration***	*Reduced viscosity****
Cacodylate buffer*	0.55_7	0.07_8
1 ml buffer, 99 ml H_2O	0.55_7	0.10_9
90 ml buffer, 10 ml dioxane	0.56_0	0.08_8
1 ml buffer, 10 ml dioxane, 89 ml H_2O	0.56_8	0.13_3

* Buffer composition is given under EXPERIMENTAL.

** Units are g/100 ml.

*** Units are deciliters per g. The reduced viscosity was calculated as $[(\eta/\eta_0) - 1]/c$, where η is the viscosity of the solution and η_0 is the viscosity of the solvent.

Degradation of thyroglobulin by papain

In water. Experiments were performed first with water as the solvent. Control experiments in which the thyroglobulin was incubated without papain showed that any protease present[44,45] as a contaminant was not sufficiently active to affect the results. In salt, with thyroglobulin in the N form, the breakdown catalyzed by papain is of the one-by-one type. In very low salt, with thyroglobulin partially in the α form, digestion with papain showed that the α form is broken down more rapidly than the N form, as LUNDGREN[46] had found before, and the sedimentation patterns suggested that the breakdown of α is of the interrupted type. In order to study breakdown of the α form alone it was necessary to find conditions either where the equilibrium favors α or, since α is broken down rapidly compared to N, where the reaction N $\longrightarrow$ α is rapid. Experiments mentioned above had shown that the addition of 10% dioxane produces the first of these effects and possibly also the second.

In 10% dioxane. Use of 10% dioxane as a solvent showed that in salt the breakdown is of the one-by-one type and that in very low salt it proceeds more rapidly and is of the interrupted type. Since variables such as pH, temperature and enzyme concentration were held constant, it is reasonable to assume that the two types of breakdown are those of papain acting on the N and α forms.

Fig. 5 shows an approximate distribution of sedimentation coefficient for the starting material in 10% dioxane and cacodylate buffer. (This same solvent was used for examination in the ultracentrifuge of all samples for which distributions were computed.) The main peak occurs at an s of 15. There is a small amount of faster sedimenting material and also some slower sedimenting material with a peak at $s = 10$. Digestion in salt (Fig. 6) shows a gradual decrease in the area of the major

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peak as well as a rapid disappearance of the peak at $s = 10$, a slower disappearance of the rapidly sedimenting material, and an increase in slowly sedimenting fragments with a peak at $s = 2$–3. Three important features of the later distributions are the absence of material between $s = 7$ and 13, the persistence of the main peak at $s = 15$ and the accumulation of non-sedimenting fragments. This last point is not brought out clearly by the differential distribution curves, since they do not show the nonsedimenting material, and so an integral distribution curve also has been included (Fig. 7) for the sample after 59 min digestion.

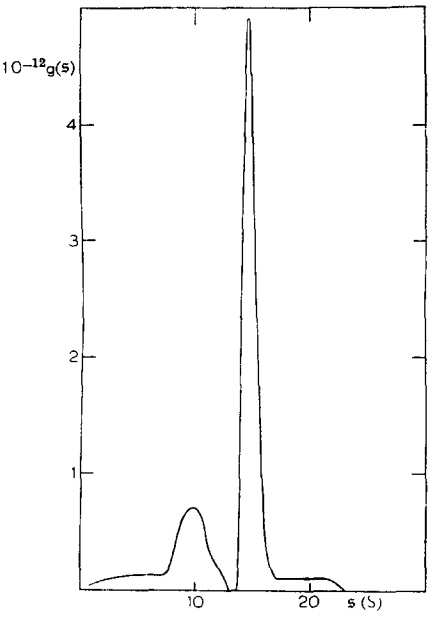

Fig. 5. An approximate distribution of sedimentation coefficient for the thyroglobulin before digestion. (The solvent is cacodylate buffer plus 10% dioxane; its composition is given under EXPERIMENTAL.)

When the digestion is carried out in very low salt, and the products of digestion are examined in salt, the distribution curves (Fig. 8) show an interrupted type of breakdown. The peak at $s = 15$ drops rapidly and intermediates are seen at all values of s less than this. Also aggregates of s greater than 18 are found. Since the denatured protein forms aggregates and proteolytic breakdown is known to produce fragments with properties like those of denatured protein[47], it is not surprising that some of these fragments aggregate. It would be interesting to convert these curves to distri-

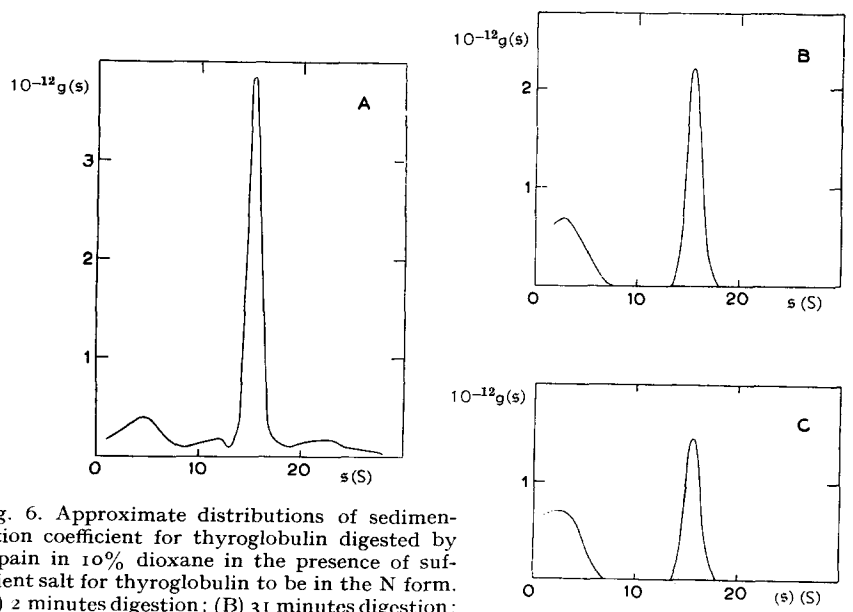

Fig. 6. Approximate distributions of sedimentation coefficient for thyroglobulin digested by papain in 10% dioxane in the presence of sufficient salt for thyroglobulin to be in the N form. (A) 2 minutes digestion; (B) 31 minutes digestion; (C) 59 minutes digestion.

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butions of molecular weight; however the aggregation in itself precludes this. Fragments produced by alkaline degradation of tobacco mosaic virus also have been found to aggregate[48].

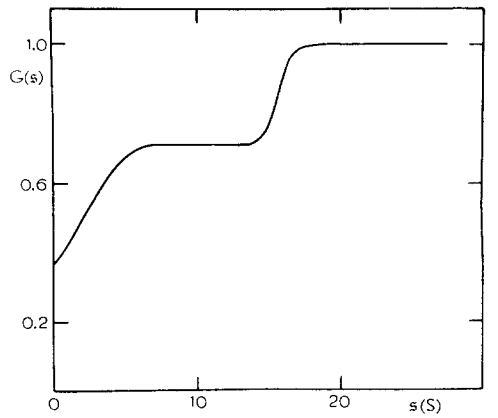

Fig. 7. The integral distribution of sedimentation coefficient corresponding to curve C of Fig. 6: thyroglobulin after 59 min digestion by papain in the presence of salt.

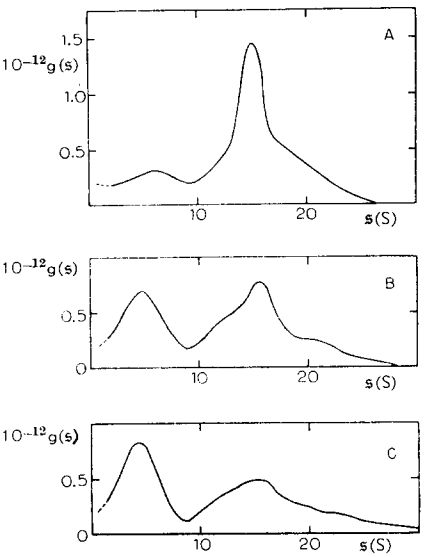

Fig. 8. Approximate distributions of sedimentation coefficient for thyroglobulin digested by papain in 10% dioxane and a sufficiently low salt concentration to produce the α form of thyroglobulin. After digestion and before examination in the ultracentrifuge, the buffer composition was increased to that of Figs. 5 and 6. (A) 2 minutes digestion; (B) 7 minutes digestion; (C) 16 minutes digestion. (The area under the curve in A is 1.00, as is the area under the curve in Fig. 5; the areas of curves B and C are 0.95 and 0.92, respectively.)

It can be seen that these distribution curves provide much information about the way in which degradation proceeds. However it should be emphasized that these curves are not fully quantitative distributions of sedimentation coefficient. No correction has been made for the dependence of sedimentation coefficients on concentration, and dependence of s on pressure may be significant when 10% dioxane is used as a solvent. Also the shape of the peak at $s = 15$ in Figs. 5 and 6 should not be taken as proof of the heterogeneity of the main component of thyroglobulin because the ratio of boundary spreading caused by heterogeneity to that caused by diffusion is probably not in the proper range[36] for satisfactory extrapolation to infinite time. (Preliminary calculations with FUJITA's equation indicate that the major component does show heterogeneity in sedimentation behavior.)

Two of the schlieren patterns from which distribution curves were calculated are included in Fig. 9. One shows the results of 31 min digestion in salt and the other shows the pattern of sedimentation after 7 min digestion in very low salt. The two types of digestion can be distinguished simply by qualitative inspection.

Effect of varying the enzyme concentration. When the one-by-one type of breakdown is found, as in the experiments in salt, there is presumably a rate-limiting stage in which one or more labile intermediates are formed. This rate-limiting step might be the conversion of N to α, even though α is present in too small amount to be

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observed by sedimentation experiments. Thus papain might not attack the native form of thyroglobulin at all. Alternatively the first labile intermediates could be produced by the action of papain on thyroglobulin. In operational terms, this is a question of whether or not the rate-limiting step in the degradation is catalyzed by the enzyme. If it is not catalyzed, then varying the enzyme concentration should not affect the rate at which native thyroglobulin is broken down. With the ultracentrifuge one can measure the amount of native thyroglobulin remaining at any time, and so an experiment was carried out in which the concentration of papain was increased by a factor of four, from $2.5 \cdot 10^{-3}$ to $1.0 \cdot 10^{-2}$ g/100 ml. The results are shown in curves A and B of Fig. 10, together with an experiment (curve C) at

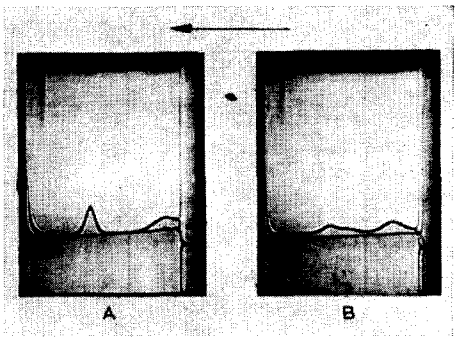

Fig. 9. Schlieren photographs showing the difference between the type of digestion in salt and in very low salt. (A) After 31 minutes digestion in cacodylate buffer plus 10% dioxane. (B) After 7 minutes digestion in very dilute cacodylate buffer plus 10% dioxane. These patterns were recorded after approximately 70 min sedimentation at 39, 460 r.p.m. The angle of the schlieren diaphragm was 65°.

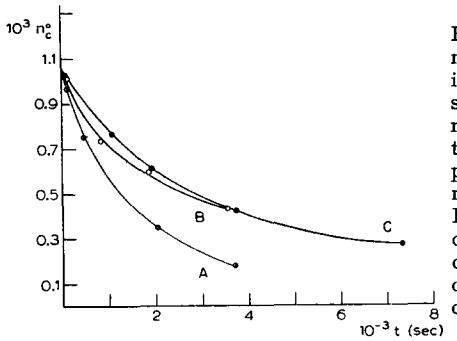

Fig. 10. The time course of the disappearance of native thyroglobulin from papain-digestion mixtures in the presence of a sufficient concentration of salt so that initially thyroglobulin is in the N form. The refractive increment n^o_c, which was measured from the area under the main peak of the sedimentation patterns, is proportional to the concentration of native thyroglobulin remaining in the digest. (A) Papain concentration — 0.01 g/100 ml; solvent — cacodylate buffer plus 10% dioxane. (B) Papain concentration — 0.0025 g/100 ml; solvent — cacodylate buffer plus 10% dioxane. (C) Papain concentration — 0.01 g/100 ml; solvent — cacodylate buffer without dioxane.

the higher enzyme concentration and in the absence of dioxane. Since the kinetics of the disappearance of N do not follow a simple rate law, it is convenient to express the results in terms of the half-time of the reaction. Increasing the enzyme concentration by a factor of four decreases the half-time from 2.2_5 to $1.1_3 \cdot 10^3$ sec, a factor of two. Thus the rate-limiting step is catalyzed, and we infer that the first labile intermediates are produced by the action of papain. It is interesting that the degradation proceeds more rapidly in the presence of dioxane.

This type of experiment was performed by LINEWEAVER AND HOOVER[49], who studied the breakdown of hemoglobin by papain and who wished to know if the rate-limiting stage of the degradation were catalyzed by papain. They found that, over a nine-fold range of enzyme concentration, their values for the digestion fell on a common graph when plotted against the product of time and enzyme concentration.

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DISCUSSION

According to these results, thyroglobulin is broken down by papain in a one-by-one manner when the N form of thyroglobulin is present and in an interrupted manner when the α form is present. This is consistent with the finding that the α form is broken down more rapidly than the N form. Thus papain attacks the α form at a rate comparable to that at which the intermediates are broken down, but in the degradation of N thyroglobulin the intermediates are attacked much more rapidly than is the native protein.

The fact that the $N \rightleftharpoons \alpha$ reaction is reversible shows that it is not necessary to break covalent bonds in order to make the protein labile to enzymic attack. Nor is it necessary to produce configurational changes of such magnitude that irreversible denaturation results, and thyroglobulin is a protein readily denatured irreversibly by urea and by acid. What then are the factors which make the α form labile and the N form resistant to attack? The answer must wait upon further research; at present the only known difference between N and α is that α has a more expanded configuration.

Several suggestions have been made recently of how hydrolysis of peptide bonds in a protein can lead to formation of labile intermediates[50,51]. SCHELLMAN AND LINDERSTRØM-LANG[50] emphasize the steric difficulties of enzymic attack when peptide bonds are in the interior of a hydrogen-bonded structure. They point out that the stability of a hydrogen-bonded structure in water depends largely on crosslinks and on tertiary structure and that breakage of a peptide bond in a loop can destroy both the loop and the tertiary structure connected with it: this leads to a more open structure of the protein and less steric resistance to enzymic attack. LASKOWSKI AND SCHERAGA[51] emphasize the purely thermodynamic stabilization of peptide bonds by formation of hydrogen-bonded structures.

Occasionally it has been suggested (for example, see ANFINSEN AND REDFIELD[52]) that the difference between the one-by-one and interrupted types of breakdown is simply a matter of the stage at which the reaction is studied. Sedimentation analysis shows clearly that this is not true of the system studied here. (This does not mean that intermediates do not exist in the one-by-one breakdown, but only that they are more labile to enzymic attack.) Figs. 6 and 8 demonstrate the difference between the two types of breakdown; this difference is seen throughout the entire period of the degradation reactions. It should be noted that the pH and temperature, as well as the concentrations of protein, enzyme and dioxane were the same in both reaction mixtures.

For a critical summary of research into the initial stages of proteolysis, the reader is referred to an article by CHRISTENSEN[47].

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SUMMARY

As shown by LUNDGREN AND WILLIAMS, hog thyroglobulin undergoes a configurational reaction, known as the N ⇌ α reaction, in solutions of low ionic strength. The reaction is reversed by adding salt. Since complete conversion to the α form is not found even in distilled water, the effects of certain agents on the N ⇌ α reaction were studied. Low concentrations of urea or acid cause irreversible denaturation, but 10% dioxane shifts the equilibrium towards the α form.

Sedimentation analysis was used to study the breakdown of thyroglobulin by papain in 10% dioxane. Action of the papain was stopped with iodoacetamide. In the presence of salt, when thyroglobulin is in the N form, degradation is of the one-by-one type defined by the absence of intermediates of large molecular weight. In solutions of very low salt concentration, in which the α form predominates, one finds the interrupted type of breakdown: the native protein disappears rapidly and large fragments are found. The pH, temperature and concentrations of papain and thyroglobulin were the same in these two sets of experiments.

Variation of the enzyme concentration showed that the rate-limiting event in the one-by-one breakdown is catalyzed by papain.

Characterization of native thyroglobulin by sedimentation and diffusion was complicated by the presence of small amounts of slower and faster sedimenting material. The sedimentation coefficient of the main component could be measured reproducibly and precisely: its dependence on total concentration is given by $s_{25,w} = 21.59\ (1 - 0.147\ c + 0.006\ c^2)$ S. This equation fits the results from twenty experiments with an average deviation of 0.22% over the concentration range $0 < c < 0.8$ g/100 ml. Studies with the Gouy diffusion method showed that the diffusing boundaries deviated markedly from Gaussian form, and the reproducibility of the experiments was less than usual.

REFERENCES

1 K. LINDERSTRØM-LANG, *Cold Spring Harbor Symposia Quant. Biol.*, 14 (1950) 117.
2 K. LINDERSTRØM-LANG, *Inst. intern. chim. Solvay, Conseil chim.*, 9th Conseil, Brussels, 1953.
3 A. TISELIUS AND I.-B. ERIKSSON-QUENSEL, *Biochem. J.*, 33 (1939) 1752.
4 B. T. CURRIE AND H. B. BULL, *J. Biol. Chem.*, 193 (1951) 29.
5 I. MORING-CLAESSON, *Biochim. Biophys. Acta*, 2 (1948) 389.
6 G. HAUGAARD AND R. M. ROBERTS, *J. Am. Chem. Soc.*, 64 (1942) 2664.
7 T. WINNICK, *J. Biol. Chem.*, 152 (1944) 465.
8 A. BELOFF AND C. B. ANFINSEN, *J. Biol. Chem.*, 176 (1948) 863.
9 C. G. POPE, *Brit. J. Exptl. Pathol.*, 20 (1939) 132, 201.
10 M. L. PETERMANN, *J. Phys. Chem.*, 46 (1942) 183.
11 W. B. BRIDGMAN, *J. Am. Chem. Soc.*, 68 (1946) 857.
12 M. L. PETERMANN, *J. Biol. Chem.*, 144 (1942) 607.
13 M. L. PETERMANN, *J. Am. Chem. Soc.*, 68 (1946) 106.
14 K. LINDERSTRØM-LANG AND M. OTTESEN, *Nature*, 159 (1947) 807.
15 K. LINDERSTRØM-LANG AND M. OTTESEN, *Compt. rend. trav. lab. Carlsberg*, Sér. chim., 26 (1949) 403.
16 M. OTTESEN, *Arch. Biochem. Biophys.*, 65 (1956) 70.
17 C. B. ANFINSEN, *J. Biol. Chem.*, 221 (1956) 405.
18 E. MIHÁLYI AND A. G. SZENT-GYÖRGYI, *J. Biol. Chem.*, 201 (1953) 189.
19 K. LINDERSTRØM-LANG, R. D. HOTCHKISS AND G. JOHANSEN, *Nature*, 142 (1938) 996.
20 K. LINDERSTRØM-LANG, *Lane Medical Lectures*, Stanford University Press, 1952.
21 M. KUNITZ AND J. H. NORTHROP, *J. Gen. Physiol.*, 17 (1934) 591.
22 F. F. NORD, M. BIER AND L. TERMINIELLO, *Arch. Biochem. Biophys.*, 65 (1956) 120.
23 H. P. LUNDGREN AND J. W. WILLIAMS, *J. Phys. Chem.*, 43 (1939) 989.
24 Y. DERRIEN, R. MICHEL AND J. ROCHE, *Biochim. Biophys. Acta*, 2 (1948) 454.
25 M. HEIDELBERGER AND K. O. PEDERSEN, *J. Gen. Physiol.*, 19 (1935) 95.
26 D. F. AKELEY AND L. J. GOSTING, *J. Am. Chem. Soc.*, 75 (1953) 5685.
27 J. R. KIMMEL AND E. L. SMITH, *J. Biol. Chem.*, 207 (1954) 515.
28 L. J. GOSTING, E. M. HANSON, G. KEGELES AND M. S. MORRIS, *Rev. Sci. Instr.*, 20 (1949) 209.
29 P. J. DUNLOP AND L. J. GOSTING, *J. Am. Chem. Soc.*, 75 (1953) 5073.
30 P. J. DUNLOP AND L. J. GOSTING, *J. Am. Chem. Soc.*, 77 (1955) 5238.
31 L. G. LONGSWORTH, *J. Am. Chem. Soc.*, 69 (1947) 2510.
32 Y. DERRIEN, R. MICHEL, K. O. PEDERSEN AND J. ROCHE, *Biochim. Biophys. Acta*, 3 (1949) 436.
33 R. L. BALDWIN, *Biochem. J.*, 65 (1957) 503.
34 R. L. BALDWIN AND J. W. WILLIAMS, *J. Am. Chem. Soc.*, 72 (1950) 4325.
35 L. J. GOSTING, *J. Am. Chem. Soc.*, 74 (1952) 1548.

[36] R. L. BALDWIN, *J. Phys. Chem.*, 58 (1954) 1081.
[37] R. L. BALDWIN, L. J. GOSTING, J. W. WILLIAMS AND R. A. ALBERTY, *Discussions Faraday Soc.*, 20 (1955) 13.
[38] M. HALWER, G. C. NUTTING AND B. A. BRICE, *J. Am. Chem. Soc.*, 73 (1951) 2786.
[39] H. FUJITA, *J. Chem. Phys.*, 24 (1956) 1084.
[40] S. SHULMAN, N. R. ROSE AND A. WITEBSKY, *J. Immunol.*, 75 (1955) 291.
[41] J. P. JOHNSTON AND A. G. OGSTON, *Trans. Faraday Soc.*, 42 (1946) 789.
[42] J. R. CANN, J. G. KIRKWOOD AND R. A. BROWN, in the press.
[43] C. TANFORD AND J. G. BUZZELL, *J. Phys. Chem.*, 60 (1956) 225.
[44] J. ROCHE AND R. MICHEL, *Ann. Rev. Biochem.*, 23 (1954) 481.
[45] J. B. ALPERS, M. L. PETERMANN AND J. E. RALL, *Arch. Biochem. and Biophys.*, 65 (1956) 513.
[46] H. P. LUNDGREN, *J. Biol. Chem.*, 138 (1941) 293.
[47] L. K. CHRISTENSEN, *Compt. rend. trav. lab. Carlsberg*, Sér. chim., 28 (1952) 37.
[48] W. F. HARRINGTON AND H. K. SCHACHMAN, *Arch. Biochem. Biophys.*, 65 (1956) 278.
[49] H. LINEWEAVER AND S. R. HOOVER, *J. Biol. Chem.*, 137 (1941) 325.
[50] J. A. SCHELLMAN AND K. LINDERSTRØM-LANG, *The Enzymes*, in the press.
[51] M. LASKOWSKI AND H. A. SCHERAGA, *J. Am. Chem. Soc.*, 78 (1956) 5793.
[52] C. B. ANFINSEN AND R. R. REDFIELD, *Advances in Protein Chem.*, 11 (1956) 73.

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THE EFFECTS OF SELECTED NITROGEN COMPOUNDS ON THE GROWTH OF PLANT TISSUE CULTURES

F. C. STEWARD, J. K. POLLARD, A. A. PATCHETT AND B. WITKOP

Department of Botany, Cornell University, Ithaca, N. Y. and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. (U.S.A.)

INTRODUCTION

Plant tissue cultures and various ancillary techniques find an increasing use in the study of growth and metabolism. In the Cornell Laboratory, tissue-culture techniques have been designed primarily to achieve control over the variables which stimulate growth by cell division and which maintain it at maximum, but quantitatively reproducible, rates. In this technique[1] tissue explants (2.5 mg) freshly removed, aseptically, from a specific region of the intact carrot root (1–2 mm from the cambium and in the secondary phloem) are stimulated to grow rapidly by as yet incompletely characterized growth factors which are to be found in the liquid endosperm of the coconut (*i.e.* coconut milk). Other similar natural fluids such as those present in immature corn (*Zea*) grains and immature *Aesculus* fruits may also be used. These growth factors are used as supplements to a basal nutrient medium which contains salts, trace elements, vitamins, and sucrose as prescribed by WHITE[2].

In the absence of the growth factors present in the coconut milk, attempts may be made to detect growth-promoting qualities, equivalent to those that are now familiar in the coconut milk. Alternatively, by the use of the complete nutrient system, in which the tissue will normally grow rapidly, the ability of a test substance to inhibit, or to retard, growth may be investigated. In addition, the same general techniques may be used to measure the interacting effects of both stimulatory and inhibitory substances when these are supplied together.

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