hemoglobin (Baltimore Biological Laboratories). Control tests with diluent devoid of lung samples yielded no microorganisms in the yolk sac culture. To identify the presence of the Legionnaires' disease organism in culture, all isolates from yolk sac tissue and bacterial cultures were stained with Gram and Giménez reagents, and by an indirect immunofluorescent technique in which human convalescent serums that contained antibodies to the Legionnaires' disease organism were used. When tested with the convalescent serums, the microorganisms exhibited green fluorescence in a coccobacillary configuration. Samples taken from viable chick embryos and 7-day-old colonies grown on bacteriologic media were examined by electron microscopy. Specimens were fixed for 48 hours in 2.5 percent glutaraldehyde, postfixed with 1 percent osmium tetroxide, and processed for routine electron microscopy.

The organisms, when grown in yolk sacs, stained variably with the Gram stain. Giménez-stained smears disclosed intracellular pleomorphic cocci, coccobacilli, beaded bacilli, and infrequent filamentous bacilli. The particles ranged in size from 300 nm to 3.7 μm. By electron microscopy, the agent exhibited a complicated intracellular morphology which consisted of replicating coccoid forms (Fig. 1A), stippled coccoid and bacillary forms (Fig. 1B), unstippled bacillary forms (Fig. 1C), shadow forms (Fig. 1D), and sporelike structures (Fig. 1F). Replicating forms, those particles that underwent reproduction by septate binary fission, and stippled coccoid and bacillary forms, those particles which contained small intracytoplasmic granules that resembled ribosomes, were located primarily within vacuoles. The unstippled bacillary forms were usually found within the cytoplasm outside vacuoles, and, by contrast, contained round electron-lucent zones, coarse granules, and one or two nucleoids. In the vicinity of sporelike structures, shadow forms were numerous. It is possible that some of the intracellular structures that we observed may represent degenerating or necrotic organisms.

The organisms grown on bacteriologic media were predominantly bacillary; but coccoid, filamentous, and pleomorphic configurations were observed (Fig. 1F). The extracellular forms exhibited a paucity of cytoplasmic organelles and reproduction by septate binary fission; nuclear membranes and mitotic figures were absent. Several morphologic components that were detected in yolk sac material were not observed in samples taken from cell-free cultures.

The Legionnaires' disease organism conforms to the morphologic criteria of a prokaryocyte. The extracellular and intracellular growth exhibited by the microorganism are compatible with transmission to humans from a form that is native to the environment, possibly involving an animal reservoir. Moreover, the observation of sporelike structures suggests possible resistance to environmental adversity. Some of the intracellular cytoplasmic coccoid and bacillary structures are morphologically similar to obligate intracellular pathogens of the order Chlamydiales, the order Rickettsiales, and small bacterial bacilli (*3*). It is possible that the organism of Legionnaires' disease, with its ability to multiply intracellularly and extracellularly, is related to an environmental microbe from which the larger obligate intracellular pathogens have evolved.

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References

1. Center for Disease Control, *Morbidity and Mortality Weekly Report* (Department of Health, Education, and Welfare Public Health Service) **25**, 30, 270 (1976).
2. Center for Disease Control, *ibid.* **26**, 9 (1977).
3. D. B. Anderson, H. E. Hopps, M. F. Barile, B. C. Bernheim. *J. Bacteriol.* **90**, 1387 (1965); R. E. Buchanon and M. E. Gibbons, Eds., *Bergey's Manual of Determinative Bacteriology* (Williams & Wilkins, Baltimore, Md., ed. 8, 1974).

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Detection of Bityrosine in Cataractous Human Lens Protein

Abstract. *Bityrosine was isolated from the insoluble protein of human cataractous lenses. Identification was based on correspondence with synthetic bityrosine with respect to chromatography, fluorescence, and ultraviolet and mass spectra. It is suggested that the compound may form cross-links with polypeptide chains in old and cataractous lenses, causing significant alteration in native protein structure.*

The basis for recent interest in fluorescent components associated with human lens protein is due to the following observations. (i) With aging, there is a dramatic increase in nontryptophan (NT) protein-bound fluorescence (*1–3*); (ii) the fluorescence appears to be associated primarily with the high-molecular-weight (HMW), yellow, insoluble protein fractions in the inner region of the lens (*1, 2, 4*); (iii) the increase in fluorescence appears to be related to increases in HMW and insoluble protein (*1, 2, 4*); and (iv) the age related production of such protein has been linked to the development of certain types of senile cataract (*5*).

It is now apparent that there is a complex group of fluorescent components associated with lens proteins. Up to the present time only one such compound, a beta carboline (*6*), has been reported.

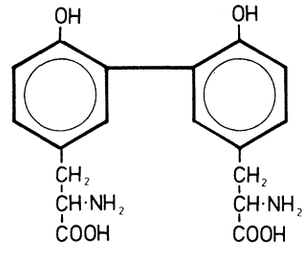

Fig. 1. Bityrosine.

Evidence has also been obtained in our laboratory suggesting the presence of anthranilic acid, a tryptophan degradation product (*7*). We now report the detection of bityrosine (Fig. 1) in the insoluble protein fraction of human cataractous lenses and confirm the unpublished suggestion of its presence in such preparations (*8*). This compound has previously only been found in connective tissue proteins (*9, 10*) such as collagen (*11*). In the lens such protein is found only in the capsular layer that surrounds the exterior of the tissue and represents approximately 1 percent of the lens weight. Nevertheless, in some experiments the capsule was eliminated before processing the lenses.

Human cataractous lenses were homogenized at 0°C in 0.01*M* tris buffer (*p*H 7.6), 0.1*M* KCl, 0.004*M* EDTA at a concentration of 1 g of lens per 10 ml of buffer and then centrifuged at 18,000*g* for 15 minutes at 4°C. After removing the supernatant, the white fraction of the precipitate was eliminated by additional washing. The remaining yellow precipitate was suspended in 0.1*M* KCl, succinylated, reduced, alkylated, and finally precipitated with acetone from an acidified solution. For general details, see Spector *et al.* (*2*). The lyophilized precipitate was suspended in 0.1*M* NH_4HCO_3 and digested in the dark with trypsin.

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Table 1. Characteristics of DE-52 fractions, bityrosine and tyrosine.

DE-52 fractions	Fluorescence spectrum (nm) (excitation/emission maxima)		Thin-layer chromatography*	
	Acid	Base†	R_F	Fluorescence visibility (long wavelength ultraviolet lamp)
Peak 2	280/310 290/350	280/310 290/350 320/400	Complex	Complex
Peak 3	325/395	305/400 ↑	0.51	Visible at basic or acidic *p*H
Peak 4	290/405	320/405 ↑	0.33	Visible only at basic *p*H
Bityrosine	290/405	320/405 ↑	0.33	Visible only at basic *p*H
Tyrosine	280/305	280/305	0.60	Not visible

*Silica gel; butanol, acetic acid, water (4 : 1 : 1). †Arrows indicate relative increase in fluorescence yield.

The solubilized digested protein was then fractionated on a DEAE cellulose (Whatman DE-52) column equilibrated with 0.05*M* NH_4HCO_3, with a stepwise gradient having increasing concentrations of NH_4HCO_3: 0.175*M*, 0.25*M*, 0.4*M*, and 1.0*M*. The peaks eluted with 0.4*M* and 1.0*M* NH_4HCO_3 contain approximately 30 and 50 percent of the NT fluorescence, and 20 and 15 percent of the 280 nm absorbance, respectively.

Both fractions were treated separately but in the same manner. Nonfluorescent components of low molecular weight were removed with a P-2 column eluted with 0.1 percent NH_4OH. The remaining material was then hydrolyzed, in the dark and under a nitrogen atmosphere, with *Streptomyces griseus* protease type VI (Sigma) for 36 to 48 hours in 0.1*M* NH_4HCO_3 at 37°C. The NH_4HCO_3 was removed by lyophilization, and, after the sample was resuspended in a small volume of water, a white insoluble material was isolated by centrifugation. The fluorescent components in this fraction were elucidated as follows.

Almost complete solubilization of this precipitate was achieved in 5*M* urea containing 50 m*M* acetate (*p*H 5.0). The preparation was applied to a DEAE cellulose (Whatman DE-52) column equilibrated with NH_4HCO_3 and then washed with water. The fluorescent material remained soluble in the absence of urea and almost complete recovery of the fluorescent components was obtained with the use of a nonlinear convex gradient between 0 and 1.0*M* NH_4HCO_3 (Fig. 2). The elution was followed by excitation and emission at 280 and 310 nm; 290 and 350 nm; and 310 and 400 nm. Fluorescence spectra were taken of the major fluorescent components. Tyrosine fluorescence (280 and 310 nm) and tryptophan fluorescence (290 and 350 nm) appeared to be localized in peak 2. The fluorescence at 310 and 400 nm was distributed in all four peaks, but, on closer inspection, the fluorescence characteristics were found to vary somewhat (see Table 1). Peak 1 was contaminated with high concentrations of urea and was not studied further. Fluorescence spectra of peaks 3 and 4 were found to have maxima in NH_4HCO_3 at 305 and 400 nm and at 320 and 405 nm, respectively. These later fractions appeared chromatographically pure.

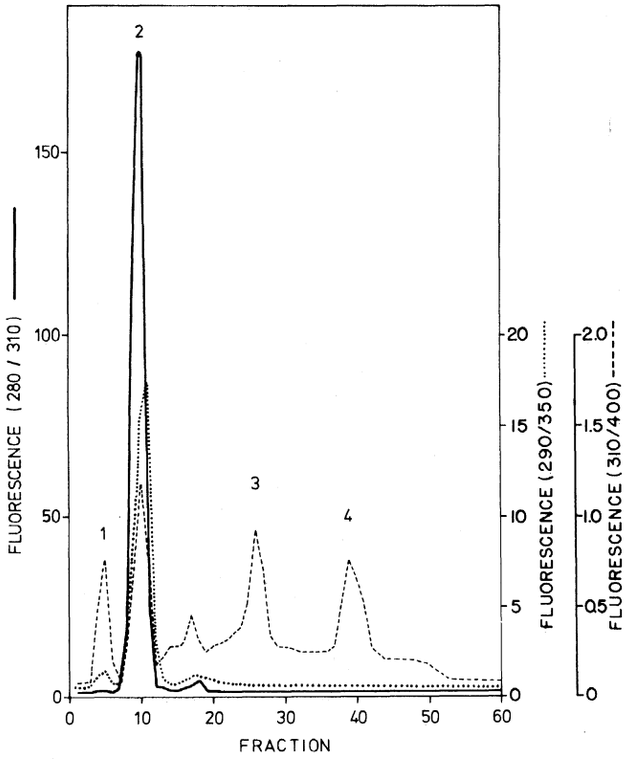

Fig. 2. DE-52 fractionation of urea-solubilized white insoluble component. See text for further information.

Peaks 2, 3, and 4 were subjected to thin-layer chromatography (TLC) on silica gel. Peak 2 contained free tyrosine and tryptophan as well as two additional spots, one of which was Folin-positive and the other Ehrlich-positive (*12*). High pressure liquid chromatography (HPLC) of peak 2 also indicated free tyrosine and tryptophan as well as three major additional fluorescent species with excitation or emission maxima of approximately 320 and 400 nm. Peaks 3 and 4 each contained one spot on TLC with the characteristics summarized in Table 1. When peak 2 was hydrolyzed with 6*N* HCl in the presence of thioglycolic acid (TGA) (*13*) and then subjected to DE-52 fractionation, three peaks were variably obtained, all of which were closely related to each other on the basis of fluorescence at different *p*H's. One of these components corresponds to the peak 4 component previously isolated, on the basis of fluorescence spectra, TLC, and elution from the DE-52 column.

The overall behavior of peak 4 suggested that this material may be bityrosine (Fig. 1). The following experiments support this conclusion. Bityrosine was synthesized by the oxidation of tyrosine (*14*) in the presence of hydrogen peroxide and horseradish peroxidase (type II, Sigma) and corresponds in mass spectra, ultraviolet absorption, and fluorescence characteristics to the previously reported synthetic product (*14, 15*). The synthetic bityrosine was purified by DE-52 and HPLC (μ-Bondapack; column, 1 by 30 cm; water; retention volume, 58 ml). The bityrosine had the same fluorescence spectra in both acid and base as peak 4 and the same increase in fluorescence in basic solution (Table 1). The mobility and fluorescent characteristics of the bityrosine and peak 4 on silica gel TLC were identical.

Both peak 4 and synthetic bityrosine were converted to the dimethyl ester and to tetrafluoroacetyl compounds (*16*), and

subjected to electron ionization (EI) mass spectrometry. Synthetic bityrosine EI; 772 (M+), 713 ($M^+ - COOCH_3$), 676, 659 ($M^+ - NH_2COCF_3$), 653 ($M^+ - COOCH_3 - HCOOCH_3$), 627 ($M^+ - NH_2COCF_3 - CH_3OH$), 563, 546 ($M^+ - 2\ NH_2COCF_3$), 492 ($M^+ - COCF_3 - CH_3OCOCHNCOCF_3$) Peak 4 EI; 659, 627, 563, 546, 492.

Because of insufficient amounts of material for EI mass spectroscopy, the molecular ion and some of the major high-molecular-weight fragments seen in the synthetic bityrosine could not be detected in peak 4. However, no other fragments appear in the 490 to 700 *m/e* (mass to charge) region of peak 4 other than those present in the spectrum of the synthetic compounds. The data clearly support the conclusion that peak 4 is 3,3-bityrosine.

The only methods reported for the synthesis of bityrosine utilize either photolysis under anaerobic conditions (*15*) or hydrogen peroxide in the presence of peroxidase (*10, 14, 17*). Both of these routes of synthesis may be available to the lens.

It has been suggested that covalent cross links may exist between polypeptide chains in the old and cataractous human lens. Bityrosine can serve as such a cross link. However the linkage could also be within the same polypeptide chain or with a free tyrosine. It is not possible from the present observations to ascertain which of these situations describe the linkage in the lens protein nor has this problem been elucidated with any other native protein where such structures have been observed.

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References and Notes

1. K. Satoh, M. Bando, A. Nakajima, *Exp. Eye Res.* **16**, 167 (1973); K. J. Dilley and A. Pirie, *ibid.* **19**, 59 (1974).
2. A. Spector, D. Roy, J. Stauffer, *ibid.* **21**, 9 (1975).
3. R. Jacobs and D. L. Krohn, *J. Gerontol.* **31**, 641 (1976).
4. R. C. Augusteyn, *Ophthalmic Res.* **7**, 217 (1975).
5. A. Spector, J. Stauffer, J. Sigelman, *Ciba Found. Symp.* **19** (new series), 185 (1973); J. A. Jedziniak, J. H. Kinoshita, E. M. Yates, L. O. Hocker, G. B. Benedek, *Exp. Eye Res.* **15**, 185 (1973); A. Spector, S. Li, J. Siegelman, *Invest. Ophthalmol.* **13**, 795 (1974).
6. J. Dillon, A. Spector, K. Nakanishi, *Nature (London)* **259**, 422–423 (1976).
7. S. Garcia-Castineiras, J. Dillon, A. Spector, in preparation.
8. R. W. Cowgill, unpublished (presented at ARVO spring meeting 1974).
9. S. O. Andersen, *Biochim. Biophys. Acta* **93**, 213 (1964).
10. F. LaBella, P. Waykole, G. Queen, *Biochem. Biophys. Res. Commun.* **30**, 333 (1968).
11. P. Waykole and E. Heidemann, *Connect. Tissue Res.* **4**, 219 (1976).
12. M. Brenner, A. Niederwieser, G. Pataki, in *Thin-Layer Chromatography. A Laboratory Handbook*, E. Stahl, Ed. (Springer-Verlag, Berlin, 1969), p. 730.
13. H. Matsubara and R. M. Sasaki, *Biochem. Biophys. Res. Commun.* **35**, 175 (1969).
14. A. J. Gross and J. W. Sizer, *J. Biol. Chem.* **234**, 1611 (1959).
15. S. S. Lehrer and G. D. Fasman, *Biochemistry* **6**, 757 (1967).
16. P. A. Cruickshank and J. C. Sheehan, *Anal. Chem.* **36**, 1191 (1964).
17. R. Aeschbach, R. Amado, H. Neukom, *Biochim. Biophys. Acta* **439**, 292 (1976).
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Isolation and Cultivation in vitro of the Actinomycete Causing Root Nodulation in *Comptonia*

Abstract. *The soil actinomycete causing formation of nitrogen-fixing symbiotic nodules on roots of the woody angiosperm* Comptonia peregrina *(L.) Coult. (Myricaceae) has been isolated from surface-sterilized root nodules after incubation and enzyme maceration. The filamentous bacterium grows slowly in pure culture on a yeast extract medium, producing sporogenous bodies which form large numbers of ovoid spores. Reinfection of sand-grown or aeroponically grown seedlings of* Comptonia *was achieved repeatedly with inocula prepared from suspensions of the* Comptonia *isolate. The same actinomycete has been reisolated from these seedling nodules. The induced nodules are highly active in the acetylene-reduction assay, and plants grow vigorously without an exogenous supply of fixed nitrogen.*

Nitrogen is the mineral nutrient which most often limits plant production. Symbiotic nitrogen fixation by root nodules of angiosperms infected with soil microorganisms is a major source for replenishment of reduced nitrogen in the living world (*1*). Members of the legume family whose roots are infected by the soil bacterium *Rhizobium* are the primary source of nitrogen fixation in agricultural systems. Some nonlegume angiosperms that form root nodules when invaded by soil actinomycetes fix atmospheric nitrogen at rates comparable to legumes, contributing major amounts of reduced nitrogen to the forests, wetlands, fields, and other natural ecosystems where they abound. In this group are 15 genera distributed among six families with more than 160 known nodulated species; all are woody dicotyledonous plants, growing as shrubs or trees scattered around the world (*2*). The most intensively studied representatives in this group are species of the genus *Alnus*.

Study of the actinomycete-induced nodule symbioses has lagged behind research on legumes largely because of the repeated failure of attempts to isolate and grow the bacterial endosymbiont in pure culture. Claims of successful isolation and culture of actinomycetes from root nodules have been published but have remained unsubstantiated and largely unaccepted by workers in the field (*3*). We report here the isolation and culture of the actinomycetous endophyte from the root nodules of the "sweet fern" *Comptonia peregrina* (L.) Coult. of the family Myricaceae. The endophyte is a filamentous bacterium that grows slowly in complex media, producing numerous spores in sporogenous bodies. A suspension of spores and fragments of filaments prepared from pure cultures of the organism inoculated into an inorganic nitrogen-free nutrient medium provided to the roots of seedlings induces prolific formation of nodules (Fig. 1A) that fix atmospheric nitrogen, as judged by acetylene-reduction tests for nitrogenase (*4*) and by the vigorous development of the plants in the absence of added nitrogen in the nutrient medium.

The commonly accepted method of producing actinomycete-induced nodules experimentally involves inoculating roots of plants grown in sand or water culture with suspensions of ground-up nodules (*5*) or applying soil suspensions taken from areas where the nodulated host plants are growing (*6*). These methods are crude and the results are unpredictable and variable. Nodule suspensions from field-grown nodules contain a wide range of soil microorganisms as well as complex products, many possibly toxic, derived from the broken plant tissues. Soil suspensions are equally complex. Quispel (*7*) discussed the problems of endophyte isolation and described methods for assessing the effectiveness of suspension inoculation. Becking (*8*) attempted to culture the endophyte from *Alnus* within proliferating callus tissue cultures derived from surface-sterilized excised nodules. No evidence for sus-

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