IgA. This assumption is supported by our failure to find goblet cell staining with FITC antibodies to light chains of chicken immunoglobulins. A compensatory increase in the numbers of IgM-containing cells was observed beneath the intestinal epithelium of these IgA-deficient birds (Figs. 1, c and d), as has been seen in humans with selective deficiency of IgA (*10*).

We also examined the effect of antibodies to μ chains on development of IgA-producing cells. Embryonic treatment with antibody to μ chains and bursectomy at hatching, as described (*11*), prevented development of IgA, IgG, and IgM in the serum (Table 1), whereas birds subjected only to bursectomy at hatching made IgM and IgG as well as controls, and most (five of nine) had normal levels of serum IgA. Spleen and intestinal tissues from five of the agammaglobulinemic birds were examined by immunofluorescence, and no cells containing IgA, IgG, IgM, or light chains were found.

These results indicate that cells capable of IgA synthesis (i) arise within the bursa of Fabricius, (ii) follow IgG committed cells in their order of migration elsewhere, and (iii) fail to develop in birds given goat antibodies to IgM heavy chain antigens as embryos and bursectomized at hatching. In several chickens bursectomized as embryos, IgM- and IgG-producing cells did not convert to IgA synthesis. The observations fit our theoretical model (*5*) in which bursal lymphocytes convert over several generations from μ chain synthesis (IgM) through γ chain (IgG) to α chain (IgA) synthesis with random cell migration at all stages of differentiation, but the possibility of direct conversion from expression of μ to α chain genes remains to be excluded. Final solution of this problem could provide information relevant to genetic mechanisms shared by many pathways of cell differentiation.

P. W. KINCADE
M. D. COOPER

Spain Immunology Research Laboratories, Departments of Pediatrics and Microbiology, University of Alabama in Birmingham, University Station, Birmingham 35294

References and Notes

1. G. J. Thorbecke, N. L. Warner, G. M. Hochwald, S. H. Ohanian, *Immunology* **15**, 123 (1968); P. W. Kincade and M. D. Cooper, *J. Immunol.* **106**, 371 (1971); Y. S. Choi and R. A. Good, *J. Exp. Med.* **136**, 9 (1972); J. M. Davie, W. E. Paul, R. Asofsky, *Fed. Proc.* **31**, 735 Abstr. (1972); R. Van Furth, H. R. E. Schuit, W. Hijams, *J. Exp. Med.* **122**, 1173 (1965); A. R. Lawton, K. S. Self, S. A. Royal, M. D. Cooper, *Clin. Immunol. Immunopathol.*, in press.
2. M. D. Cooper, W. A. Cain, P. J. Van Alten, R. A. Good, *Int. Arch. Allergy* **35**, 242 (1969).
3. P. W. Kincade, A. R. Lawton, D. E. Bockman, M. D. Cooper, *Proc. Nat. Acad. Sci. U.S.A.* **67**, 1918 (1970).
4. P. W. Kincade, K. S. Self, M. D. Cooper, *Fed. Proc.* **31**, 654 Abstr. (1972); *Cell. Immunol.*, in press.
5. M. D. Cooper, P. W. Kincade, A. R. Lawton, in *Immunologic Incompetence*, B. M. Kagan and E. R. Stiehm, Eds. (Year Book Medical, Chicago, 1971), pp. 81–104; M. D. Cooper, A. R. Lawton, P. W. Kincade, *Clin. Exp. Immunol.* **11**, 143 (1972).
6. A. R. Lawton, R. Asofsky, M. B. Hylton, M. D. Cooper, *J. Exp. Med.* **135**, 277 (1972); D. D. Manning and J. W. Jutila, *J. Immunol.* **108**, 282 (1972).
7. A. M. Lebacq-Verheyden, J. P. Vaerman, J. F. Heremans, *Immunology* **22**, 165 (1972); J. Bienenstock, D. Y. E. Perey, J. Gauldie, B. J. Underdown, *J. Immunol.* **109**, 403 (1972).
8. G. Mancini, J. P. Vaerman, A. D. Carbonara, J. F. Heremans, in *Peeter's XI Colloquium on Protides of the Biological Fluids* (Elsevier, Amsterdam, 1964).
9. P. A. Crabbe, A. O. Carbonara, J. F. Heremans, *Lab. Invest.* **14**, 235 (1965); D. R. Tourville, J. Bienenstock, T. B. Tomasi, *J. Exp. Med.* **129**, 411 (1969).
10. P. A. Crabbe and J. F. Heremans, *Gut* **7**, 119 (1966).
11. In these experiments, 2 mg, as described (*3*), of purified goat antibodies to chicken μ chains were given intravenously on day 13 of egg incubation and surgical bursectomy at hatching was followed by treatment with 1 mg of antibody to μ chains.
12. We thank Dr. A. R. Lawton, III, for discussion, and W. E. Gathings and J. N. Henson for technical assistance. Supported by NIH grants CA13148 and 1 F02 A152040-01 (to P.W.K.), and the American Cancer Society, Alabama Division.

5 September 1972; revised 24 October 1972

Isolation and Structure Determination of Blepharismin, a Conjugation Initiating Gamone in the Ciliate Blepharisma

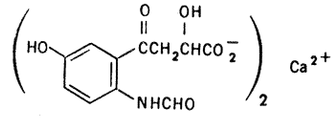
Abstract. *One of the gamones (gamone II) which are effective for the induction of conjugation in* Blepharisma intermedium *has been isolated in a crystalline form and designated as blepharismin. From the result of chemical and spectroscopic investigations, in which x-ray crystallographic analysis was used as a definitive tool, blepharismin has been found to have the structure of calcium 3-(2′-formylamino-5′-hydroxybenzoyl)lactate.*

Control of conjugation in Protozoa by specific chemical substances has long been assumed (*1*), although their unequivocal identification has been hampered mainly because they are hard to obtain in a soluble form. Recently, the complementary mating types (I and II) of *Blepharisma intermedium* were found to excrete two kinds of gamone (I and II) into the medium, and their characterizations have been reported (*2*, *3*). Of these, gamone II (factor R) (*3*) is responsible for inducing mating type I to conjugate. This report deals with the isolation of gamone II, designated as blepharismin, in a crystalline form and the elucidation of its structure.

Cell-free fluid with gamone II activity (*3*) was concentrated in vacuo to a small volume. The precipitate was removed by filtration. The filtrate was evaporated to dryness and the residue was extracted with absolute ethanol to give partially purified material, which exhibited nine spots under ultraviolet light on the paper chromatogram (Toyo Roshi No. 51; developing solvent, tertiary butanol : concentrated ammonium hydroxide : water, in the proportions 3 : 1 : 1). Of these, only a greenish yellow spot (R_F ratio = 0.56) was responsible for the gamone II activity. Further purification was achieved by column chromatography with cellulose powder (Toyo Roshi 100 to 200 mesh; 75 percent *n*-propanol as eluting solvent) and preparative paper chromatography (Toyo Roshi No. 131). Blepharismin crystallized from water as pale yellow prisms, which exhibited gamone activity at a concentration of 10^{-9} g/ml on mating type I suspended at a density of 500 cells per milliliter. The yield was 3.68 mg from 48 liters of the cell-free fluid with 80 units/ml of gamone II activity.

Blepharismin is a highly polar substance and did not melt below 300°C. The ultramicroanalysis of a partially dried sample (70°C at 1 mm-Hg for 8 hours) showed carbon, 44.70; hydrogen, 4.66; and nitrogen, 4.68 percent. The result of oxygen analysis indicated the presence of some other element, which was determined to be calcium by emission analysis and electron spectroscopy for chemical analysis (ESCA) (observation of $2P_{1/2}$ and $2P_{3/2}$ bands). Undried crystals were estimated to have the molecular weight of 670/2 = 335 (x-ray method) and gave the analytical values of 4.26 and 6.48 percent for nitrogen and calcium (atomic absorption method) respectively. These results, combined with the structural evidence (see below), are best interpreted by assuming that blepharismin has the molecular formula $C_{11}H_{10}NO_6 \cdot 0.5\ Ca \cdot$

1.5 H_2O (calculated values: C, 44.09; H, 4.37; N, 4.69; Ca, 6.70 percent) in the partially dried state and $C_{11}H_{10}NO_6 \cdot 0.5\ Ca \cdot xH_2O$ (x is approximately 3.5) in the undried form. Blepharismin exhibited broad infrared bands due to hydroxyl and carbonyl groups at 3280 and 1662 cm^{-1}, respectively, as well as a peak due to a carboxylate group at 1595 cm^{-1}. In the ultraviolet region it showed absorption maxima at 234 (ε, 22400), 259 (shoulder, 9500), and 340 nm (4600) in water; these underwent a bathochromic shift on the addition of alkali to 244 (ε, 24600), 280 (shoulder, 8900), and 370 nm (4600), respectively. This evidence indicated that blepharismin is a phenol which has a *meta*-substituted carbonyl functional group. The analysis of the nuclear magnetic resonance spectrum (in D_2O solution, with a Fourier transformer) substantiates more clearly the substitution pattern of the phenol ring. Blepharismin exhibited four resonance signals in the aromatic proton region; the chemical shift, δ, was 7.05 (double doublet, $J = 8.7$ and 3 hertz); 7.29 (doublet, $J = 3$ hertz); 7.75 (doublet, $J = 8.7$ hertz); and 8.21 (singlet) parts per million. Taking the values of the coupling constant and the additivity rule for substitution effects in chemical shifts (*4*) into consideration, we conclude that blepharismin is a 3,4-disubstituted phenol (a carbonyl group in the 3 position). The singlet at the lowest field could be due to a N- or O-formyl proton or a N-heterocycle proton, but the discrimination was not possible at this stage. Scarcity of the material available prevented further studies by chemical means, and the complete structure of blepharismin was deduced from x-ray crystallographic analysis. The result of this analysis (*5*) is reproduced in Fig. 1. Thus, blepharismin has the structure of 3-(2′-formylamino-5′-hydroxybenzoyl)lactate as formulated below.



Blepharismin is the first gamone in Protozoa of which the molecular structure has been revealed. Its structure has several interesting features from the biochemical viewpoint. Clearly, blepharismin is biogenetically involved in the metabolism of tryptophane. In particular, it has a close relationship with kynurenine, which plays an important role in the formation of brown pigments in insects (*6*). The presence of the phenolic hydroxyl group at the 5′ position is consonant with the fact that this position of the indole ring is most vulnerable to oxidation, both chemically and biologically (*7*). Serotonin and melatonin are the indole derivatives of physiological significance (*7*, pp. 438–439) which are similarly oxygenated on the ring. The replacement of the α-amino group in kynurenine by the hydroxyl group is another structural feature in blepharismin. This represents a rare case in which a lactic acid type of compound displays a significant biological activity, although the oxidation of a α-amino group to a hydroxyl group is a very general biological process. Finally, blepharismin is the calcium salt of a carboxylic acid in the peculiarly coordinated form. The calcium coordination plays an important role in the stabilization of the β-carboxy-β'-hydroxyketone structure. However, further study is needed to conclude that the presence of calcium is indispensable for gamone activity.

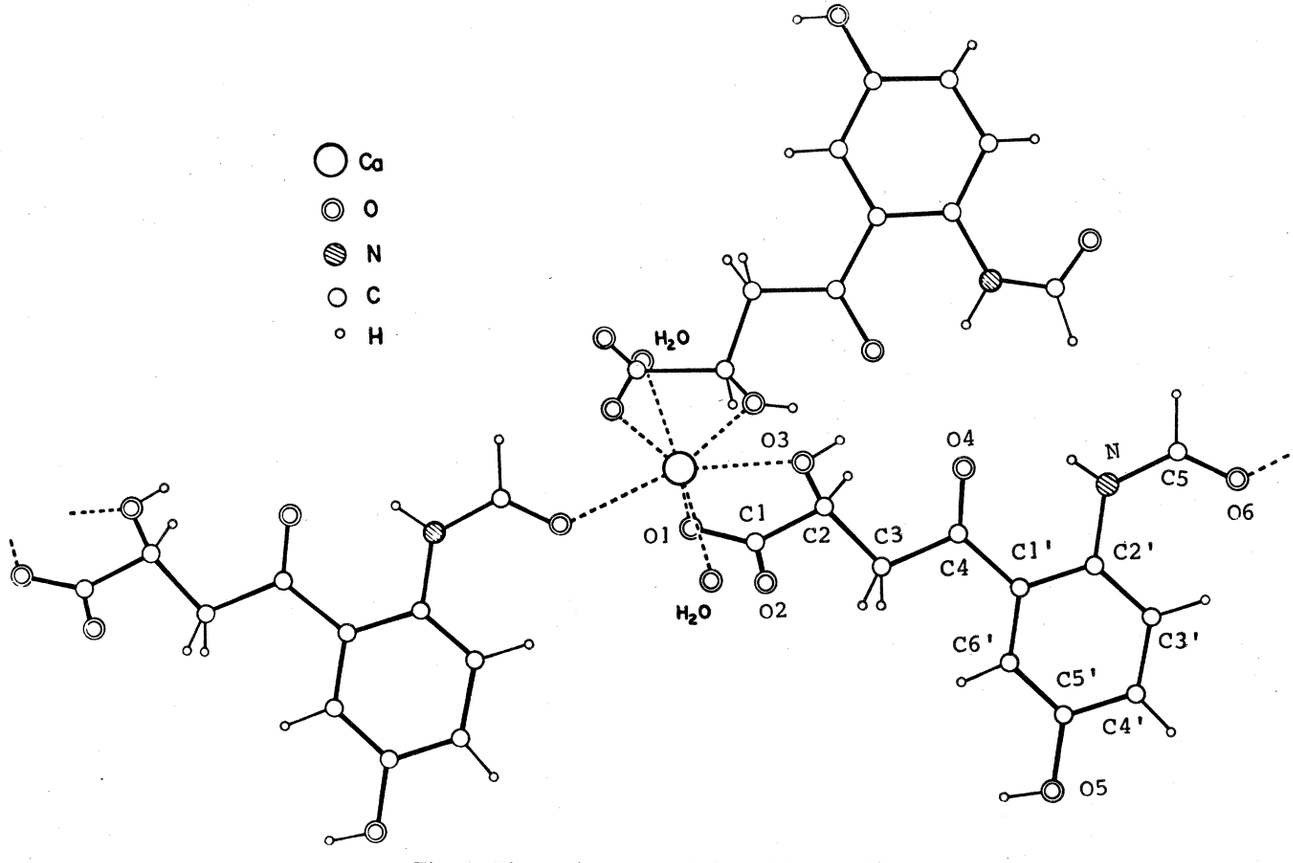


Fig. 1. The environment of the calcium cation.

So far, the structure of several gamones in water molds has been clarified (*8*). It is of great interest that a gamone in Protozoa structurally belongs to a novel type of compound which has general biochemical importance. Elucidation of the structure of blepharismin is expected to facilitate the molecular study of the conjugation mechanism in *Blepharisma*.

TAKASHI KUBOTA
TAKASHI TOKOROYAMA
Faculty of Science,
Osaka City University,
Osaka 558, Japan

YOSHIKO TSUKUDA
HIROZO KOYAMA
Shionogi Research Laboratory,
Shionogi and Co., Ltd.,
Osaka 553

AKIO MIYAKE
Max-Planck-Institut für Molekulare
Genetik, Berlin 33, Germany

References and Notes

1. T. M. Sonneborn, *Proc. Nat. Acad. Sci. U.S.A.* **23**, 378 (1937); *J. Exp. Zool.* **113**, 87 (1950); C. B. Metz, in *Sex in Microorganisms*, D. H. Wenrich, Ed. (AAAS, Washington, D.C., 1954), p. 284.
2. A. Miyake, *Proc. Jap. Acad.* **44**, 837 (1968).
3. ——— and J. Beyer, in preparation.
4. L. M. Jackman and S. Sternhell, *Application of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry* (Pergamon, Oxford, 1969), p. 202.
5. Y. Tsukuda and H. Koyama, in preparation.
6. H. Kikkawa, *Advan. Genet.* **5**, 107 (1953); A. Buetnandt, *Jahrb. Max-Plank-Ges. Ford. Wiss.* (1951), p. 160.
7. R. J. Sundberg, *Chemistry of Indoles* (Academic Press, New York, 1970), pp. 308–309.
8. L. Cagliotti, G. Cainelli, B. Camerino, R. Mondelli A. Prieto, A. Quilico, *Tetrahedron* (Suppl.) (No. 7), 175 (1966); T. Reschke, *Tetrahedron Lett.* **1969**, 3435 (196 W. H. Nutting, H. Rapopport, L. Machlis, *J. Amer. Chem. Soc.* **90**, 6434 (19(G. P. Arsenault, K. Biemann, A. W. Barksdale, T. C. McMorries, *ibid.*, p. 5635.
9. Part of this work (T.K. and T.T.) was supported by grants for scientific research from the Ministry of Education and a research grant from the Naito Foundation. We thank K. Matsumoto of Shionogi Research Institute for his assistance in the production of the gamone preparation.

18 July 1972

Embryonic Chick Intestine in Organ Culture: Response to Vitamin D_3 and Its Metabolites

Abstract. *Embryonic chick intestine maintained in organ culture responded to vitamin D_3 and its metabolites 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol by synthesis of calcium-binding protein and enchanced calcium-45 uptake. The dihydroxy metabolite was by far the most potent inducer of the protein and also acted more rapidly than vitamin D_3 to stimulate isotope uptake. Despite its lower potency, vitamin D_3 itself was effective.*

Vitamin D_3 induces de novo synthesis of a calcium-binding protein (Ca-BP) and enhances uptake and mucosal to serosal transport of Ca in embryonic chick intestine maintained in organ culture (*1*, *2*). Much correlative evidence supports a role for Ca-BP in the vitamin D–mediated intestinal transport of Ca in vivo (*3*). Also, direct evidence was obtained with the organ culture technique: absorption and transport of Ca was enhanced in intact, everted duodena maintained with purified Ca-BP but no vitamin D_3 in the culture medium (*2*).

With improvements in the culture technique, mucosal structure was well preserved after 48 hours of incubation in a defined, serum-free medium (*4*). The marked similarity between responses to vitamin D in vivo and in this system indicates that the metabolic fate and mode of action of the vitamin can validly be studied in the organ culture. This system may be of use in understanding the physiological significance of active metabolites of vitamin D_3 (cholecalciferol): 25-hydroxycholecalciferol (25-OHD_3), formed in the liver (*5*) and kidney (*6*) from vitamin D_3; and 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$], formed in the kidney from 25-OHD_3 (*7*). I report here the relative effectiveness of vitamin D_3 and its metabolites. Although 1,25-$(OH)_2D_3$ was extremely potent, vitamin D_3 itself was effective.

Entire duodena from four 20-day chick embryos (300 to 400 mg of tissue) were slit open and laid with mucosa upward on a 60-mesh stainless steel grid (51 by 63.5 mm on 6.35-mm legs) inside a petri dish. The dish contained McCoy's 5A medium without serum (*8*), with 1.25 m*M* Ca (except when noted otherwise) and nystatin (100 unit/ml). The guts on grids, cultured for 48 hours in a humidified incubator (37.5°C) continuously gassed with 5 percent CO_2 in air, produced up to ten times as much Ca-BP in response to 26 μM vitamin D_3 as did tissue in the original immersion culture method (*1*). After culture, the guts were either homogenized before Ca-BP measurement by radial immunoassay (*1*), or ^{45}Ca uptake was measured during a 30-minute incubation in a buffer solution (*1*). As measured here, ^{45}Ca uptake reflects the first step in the transport of Ca across the intestine, namely, accumulation of Ca by the cell. The validity of this method has been established (*9*, *10*) and the dependence of uptake on vitamin D was shown in vivo (*9*) and in this organ culture system (*1*).

The results of culturing duodena in the presence of vitamin D_3, 25-OHD_3, or 1,25-$(OH)_2D_3$ (*8*) are shown in Table 1. The minimum effective concentrations of either vitamin D_3 or 25-OHD_3 was only 6.5 n*M* for induction of detectable Ca-BP synthesis and 65 n*M* for enhancement of ^{45}Ca uptake. At 65 n*M*, 25-OHD_3 was about 1.4 times as effective as vitamin D_3 in inducing Ca-BP synthesis and twice as effective in stimulating ^{45}Ca uptake. Although the cultured duodena responded to relatively low concentrations of both

Table 1. Relative potency of vitamin D_3 metabolites in stimulating Ca-BP synthesis and ^{45}Ca uptake in embryonic chick intestine in organ culture. Values are mean ± standard error. Duodenal tissue was cultured for 48 hours with sterol in the culture medium. The tissues were then analyzed for Ca-BP content or transferred to a ^{45}Ca-containing buffer solution and incubated for 30 minutes at 37°C (*1*). Uptake of ^{45}Ca was determined by liquid scintillation counting of duodena in Nuclear-Chicago solubilizer. Relative potency of Ca-BP synthesis is 100 times the average Ca-BP concentration induced by a particular sterol divided by the average Ca-BP concentration induced by vitamin D_3. Relative potency of ^{45}Ca uptake is 100 times the average net increase in ^{45}Ca uptake stimulated by a particular sterol divided by the average net increase in ^{45}Ca uptake stimulated by vitamin D_3.

Sterol (n*M*)	Ca-BP (μg per 100 mg of tissue)			^{45}Ca uptake (% of control)		
	D_3	25-OHD_3	1,25-$(OH)_2D_3$	D_3	25-OHD_3	1,25-$(OH)_2D_3$
0 (Control)	0	0	0	100	100	100
0.65			8.4			100 ± 12
6.5	1.5 ± 0.2	0.8 ± 0.1	21.0	103 ± 8	99 ± 8	101 ± 3
65	3.0 ± 0.5	4.3 ± 0.3	28.8	120 ± 12*	140 ± 8*	144 ± 6*
	*Relative potency of 65 n*M *sterol*					
	100	143	960	100	200	220

* $P < .01$ by Student's *t*-test applied to uptake data expressed as percentage of ^{45}Ca dose accumulated per 100 mg of tissue.