IN VITRO SYNTHESIS OF THE FIRST COMPONENT OF COMPLEMENT BY GUINEA PIG SMALL INTESTINE

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Modern methods for assay of individual complement (C′) components on a molecular basis provide a rational approach to the study of the biosynthesis of C′ components. The specificity, sensitivity, and accuracy of these new methods and the availability of radioactively labeled amino acids and of inhibitors of protein synthesis[1, 2] have made it possible to obtain the evidence, presented in this report, that the first component of complement (C′ 1) is synthesized by isolated guinea pig small intestine. This is the first evidence for incorporation of an amino acid into a hemolytically active complement component during *in vitro* synthesis.

Materials and Methods.—Preparation of organs: Male or female guinea pigs (strain 2, Hartley and NIH-Hartley hybrids), each weighing approximately 750 gm, were killed by removing 10–15 ml of blood by heart puncture. The alimentary tract was removed immediately, opened lengthwise, and washed at 0°C in Ringer's-lactate solution with 5% glucose (Abbott) containing 50 units penicillin and 50 μg streptomycin per ml (RL buffer). At this stage of preparation, segments of esophagus, stomach, small intestine, cecum, colon, liver, spleen, lung, testes, and kidney contained C′1 activity, probably due to the presence of serum. In all experiments, this initial level of C′1 activity was eliminated by incubation of each of the tissues for 40 min at 37°C in 0.01 *M* EDTA (ethylenediaminetetraacetic acid)-veronal buffer (pH 7.5) containing 0.1% gelatin.[3] The segments were then washed twice in ice-cold RL buffer and tested for C′1 activity.

Incubation media: Tissues were incubated at 30°C or 4°C in either RL buffer or medium 199, also containing 50 units penicillin and 50 μg streptomycin per ml. For inhibition studies, fresh stock solutions were prepared daily containing 100 μg of inhibitor per ml of medium. Puromycin or actinomycin D each were used at a concentration of 10 μg per ml of incubation medium.

The activity of partially purified C′1a[4] (the activated form of C′1) was unaffected by incubation of C′1a with these inhibitors. Furthermore, assays of C′1a yielded the same results in the presence and absence of these inhibitors.

Assay of C′1 activity: The assay for C′1 activity is based on the transfer of C′1 from donor tissue to appropriate recipient cells.[5] The recipient cells (EAC′4)[6] consist of sheep erythrocytes (E), sensitized with Forssman antibody (A) and the fourth component of complement (C′4). Tissue fragments measuring approximately 5 × 5 mm (average wet weight 34 mg) were incubated with 0.5 ml of EAC′4 (1.5×10^8 cells per ml) for 10 min at 30°C. The second component of complement (C′2) and C′-EDTA (to supply the components of the C′3 complex) were added, and the number of C′1 molecules transferred, measured as C′1a, was calculated from the degree of hemolysis.[5] Control mixtures consisting of tissue fragments and EAC′4 or E and veronal-buffered saline were incubated as in the assay for C′1a. In the absence of C′2 and C′EDTA there was no detectable hemolysis in these control samples. Diluents for measurement of C′1a activity were veronal-buffered saline[3] and sucrose-veronal buffer[7] (VB-sucrose).

Measurement of radioactivity: L-lysine, uniformly labeled with C^{14} (specific activity 240 mc/mmole, lot # 6601) was obtained from Schwarz BioResearch, Inc. The radiolabeled lysine was used at a final concentration of 1 μc/ml. Aliquots of 0.5 ml of the samples to be counted were dried on planchets at 60°C. Radioactivity was measured in a Tracerlab gasflow counter, set to measure the time required for 100 counts.

Results.—Site of C′1 synthesis: Slices of liver, spleen, lung, kidney, testes, and segments of the alimentary tract were incubated separately in RL buffer or medium 199 at 4°C. C′1 activity was determined at timed intervals. Small bowel was the only tissue capable of reproducible C′1 synthesis under these conditions.

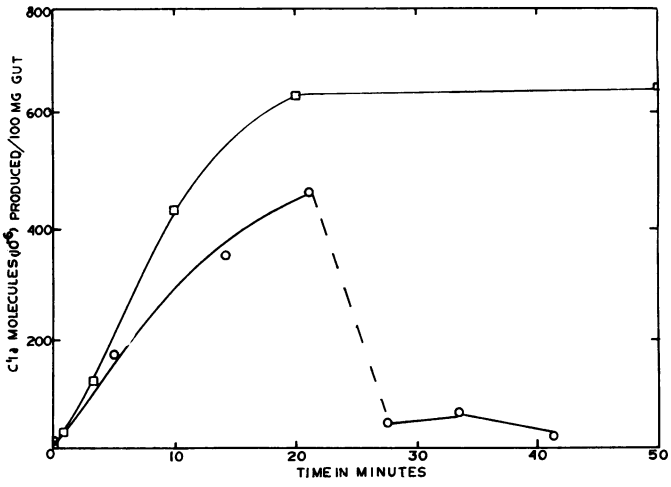

FIG. 1.—Synthesis of C'1 by small intestine at 30°C. ○, In RL buffer; □, in medium 199.

Germ-free guinea pig small intestine showed a similar capacity for C'1 production. Occasionally, tissues other than the small intestine showed an increase in C'1 activity. This increase, however, was relatively low, unsustained, and not reproducible for a given tissue. These data are summarized in Table 1.

Effect of incubation medium: A section of small bowel was incubated in medium 199 and an adjacent segment in RL buffer, both at 30°C. C'1 activity was measured at zero time and at intervals thereafter. Tissues incubated in medium 199 were capable of quantitatively greater, more rapid and prolonged C'1 synthesis than tissues incubated in RL buffer (see Fig. 1).

At 4°C, the rate and extent of C'1 synthesis were also greater in medium 199 than in RL buffer (see *small intestine*, Table 1). The rate of C'1 synthesis at 30°C in either medium was approximately 50–100 times the rate of synthesis at 4°C.

Effect of actinomycin D on C'1 synthesis: Segments of washed guinea pig small intestine (about 5 cm long) were incubated at 30°C or 4°C in media containing 10

TABLE 1

AMOUNT OF C' 1 IN DIFFERENT TISSUES INCUBATED AT 4°C IN RINGER'S-LACTATE (RL) BUFFER OR IN MEDIUM 199

	Time in Hours							
	0		24		48		72	
	(No. of C'1 molecules/100 mg tissue fragment) × 10^{-6}							
	199	RL	199	RL	199	RL	199	RL
Esophagus	NT	1	NT	6.3	NT	NT	NT	NT
Stomach	18.4	18.5	15	14.6	18.2	2.9	NT	NT
Small intestine	10.1	1	376	149	475	298	NT	325
Cecum	7.6	6.3	3.2	12.2	1.6	9.0	NT	NT
Colon	11.9	11.9	8.8	6.1	1.8	1	NT	NT
Liver	17.5	3.8	20.0	25.7	17.1	15.5	NT	NT
Spleen	7.6	29	4.5	25	9.0	20	NT	NT
Kidney	2.5	1	5.8	62	3.6	7.8	NT	NT
Testes	NT	5.6	NT	1	NT	NT	NT	NT
Lung	38.9	6.0	4.7	1	4.5	NT	NT	NT
Germ-free small intestine	5.6	NT	465	NT	484	NT	517	NT

NT = not tested.

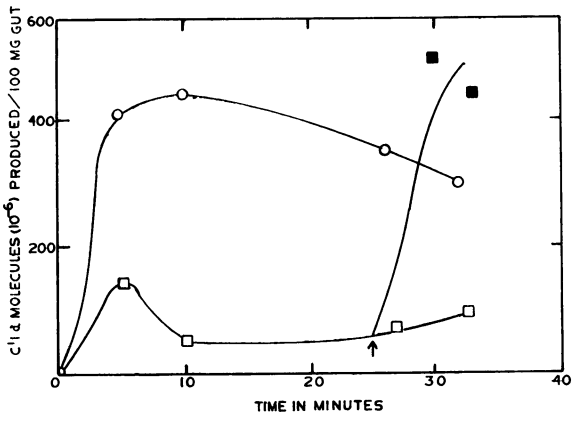

FIG. 2.—Effect of actinomycin D on the synthesis of C'1 at 30°C. O, Medium 199 lacking actinomycin D; □, medium 199 + actinomycin D; ■, tissue removed at ↑ and reincubated in medium 199 lacking actinomycin D.

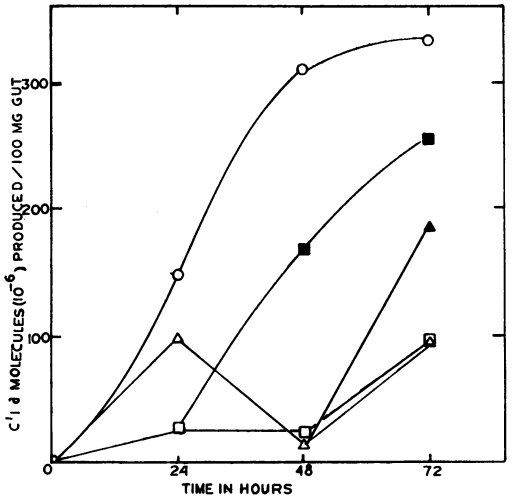

FIG. 3.—Effect of puromycin and actinomycin D on C'1 synthesis at 4°C in RL buffer. O, Control lacking inhibitors; □, puromycin; △, actinomycin D; ■, removed from puromycin at 24 hr and reincubated in RL buffer; ▲, removed from actinomycin D at 48 hr and reincubated in RL buffer.

μg actinomycin D per ml. Control segments were incubated in media lacking actinomycin D, and experimental as well as control samples were assayed for C'1 activity at convenient times. In the presence of actinomycin D, a brief period of nearly normal synthesis was followed by inhibition of C'1 synthesis. At the time of maximal inhibition, tissue transferred to a medium lacking actinomycin D resumed production of C'1 (see Figs. 2 and 3).

Effect of puromycin on C'1 synthesis: Puromycin at a concentration of 10 μg per ml immediately inhibited C'1 synthesis by small bowel in medium 199 at 4°C or RL buffer at 30°C and 4°C. Puromycin inhibition of C'1 synthesis was re-

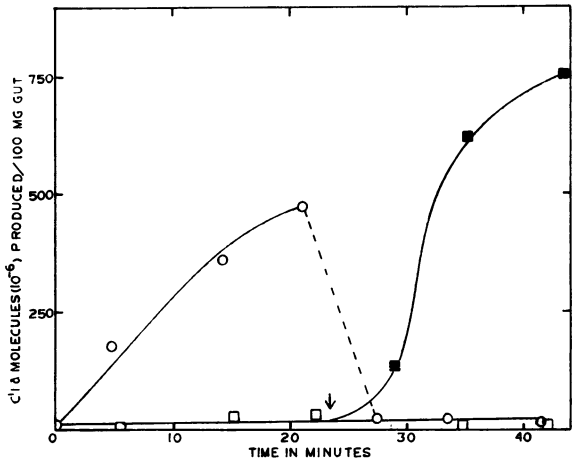

FIG. 4.—Effect of puromycin on the synthesis of C'1 at 30°C in RL buffer. ○, No puromycin; □, puromycin; ■, tissue removed at ↓ and reincubated in RL buffer lacking puromycin.

versed by transferring a portion of the gut to RL buffer lacking puromycin (see Figs. 3 and 4). On the other hand, in medium 199 at 30°C, puromycin in concentrations up to 50 μg per ml caused an apparent enhancement of C'1 synthesis (see Table 2).

In vitro incorporation of C^{14}-lysine into C'1: A piece of guinea pig small intestine was divided into three approximately equal segments. Two of the segments were used for controls (see below), and one of the segments was incubated for 18 hr at 4°C in RL buffer containing uniformly labeled C^{14}-lysine (1 μc/ml) and divided into four portions. One portion was tested for C'1a activity. The second portion was mixed with 1.0 ml of EAC'4 (1×10^9 cells/ml), the third portion with 1.0 ml of EA (1×10^9 cells/ml), and the fourth portion with E (1×10^9 cells/ml). The diluent for the preparations containing red cells was veronal buffer. Each of the preparations was incubated for 10 min at 37°C, the portions of intestine were removed, and the red cells were collected by centrifugation and washed twice in VB-sucrose buffer. Each red-cell preparation was divided into two equal parts after resuspension in 2 ml of VB-sucrose buffer. One part was mixed with 10 ml of 0.01 *M* EDTA-veronal buffer and the other with 10 ml of VB-sucrose buffer. The EA and E preparations were treated in a similar manner. After incubation of the mixtures for 10 min at 37°C, the cells were washed twice in VB-sucrose buffer. The cells were resuspended in 1.1 ml of unlabeled partially purified C'1a (5×10^{12} molecules per ml) and incubated for 30 min at 30°C. The preparations were centrifuged and the radioactivity of the supernatant fluids was measured.

TABLE 2

EFFECT OF PUROMYCIN ON SYNTHESIS OF C'1 BY SMALL INTESTINE AT 30°C IN MEDIUM 199

	Time of Incubation (Min)			
	0	2	6	28
	(No. of C'1 molecules/tissue fragment) $\times 10^{-6}$			
199	13	26	104	99
199 + Puromycin		267	235	481

TABLE 3

INCORPORATION OF C^{14}-LYSINE INTO C'1 BY SMALL INTESTINE

Recipient cells		Treatment of cells before elution with "cold" C'1a	Cpm/tissue fragment (eluted)
		18 Hr in C^{14}-Lysine	
1	EAC'4	VB sucrose	47
2	EAC'4	EDTA	27
3	EA	VB sucrose	75
4	EA	EDTA	19
5	E	VB sucrose	9
6	E	EDTA	10
		0 Time Control	
7	EAC'4	VB sucrose	6
8	EAC'4	EDTA	3
9	EA	VB sucrose	8
10	EA	EDTA	4
11	E	VB sucrose	5
12	E	EDTA	0
		18-Hr Control	
13	EAC'4	VB sucrose	4
14	EAC'4	EDTA	4
15	EA	VB sucrose	3
16	EA	EDTA	4
17	E	VB sucrose	2
18	E	EDTA	6
		Cells Exposed Directly to C^{14}-Lysine-in Absence of C'1	
19	EAC'4	VB sucrose	6
20	EAC'4	EDTA	3
21	EA	VB sucrose	2
22	EA	EDTA	6
23	E	VB sucrose	7
24	E	EDTA	6
		Partially Purified C'1a Mixed with C^{14}-Lysine	
25	EAC'4	VB sucrose	8
26	EAC'4	EDTA	8
27	EA	VB sucrose	6
28	EA	EDTA	3
29	E	VB sucrose	11
30	E	EDTA	5
31	No cells	VB sucrose	4
32	No cells	EDTA	1

The design of this experiment was based on the facts that C'1 is fixed by EA and by EAC'4, but not by E, and that the binding of C'1 to sensitized cells is reversible. Thus, the addition of a large excess of unlabeled ("cold") C'1a to cells containing labeled C'1 on their surface, causes a shift in the equilibrium between sensitized cells and labeled C'1. As a result of this shift, most of the labeled C'1 will be eluted and found in the fluid phase.

0.1 ml of medium (0.1 μc) contains 7.08×10^4 cpm (counted for 10 min).

One of the controls consisted of one of the three initial segments of intestine. This segment was incubated for 5 min at 4°C in RL buffer containing C^{14}-lysine and then washed free of radiolabel (zero time control). The remaining segment of the original piece of intestine was incubated in RL buffer for 18 hr at 4°C and then for 5 min at 4°C in the presence of C^{14}-lysine (18-hr control). These two segments then were treated in exactly the same way as the first segment.

Additional controls were set up to determine whether red cells, cellular intermediates (EAC'4 and EA), or C'1a bind C^{14}-lysine in such a way as to permit release of label in the presence of unlabeled C'1a. The results of these experiments are presented in Table 3. Significant amounts of radioactivity could be eluted only from EA and EAC'4 which had been exposed to segments of gut incubated for 18 hr in the presence of C^{14}-lysine.

Discussion.—Previous efforts to demonstrate the synthesis of complement components *in vitro* have yielded equivocal results. Thorbecke *et al.*[8] and Hochwald *et al.*[9] have demonstrated the capacity of various organs to incorporate C^{14}-labeled amino acids into β-globulins capable of interaction with anticomplement antibodies. It is not known, however, whether these β-globulins possessed hemolytic activity. The work of Siboo and Vas[10] on the synthesis of C'2, C'4, and C'3 complex suffered from inability to distinguish between release of preformed complement components and synthesis. Furthermore, they were unable to demonstrate incorporation of amino acid into hemolytically active complement components. In this report, evidence is presented that the first component (C'1) of guinea pig complement is synthesized in the tissues of the small intestine. This evidence is based on the finding that during incubation of intestine in the presence of C^{14}-lysine the

amino acid is incorporated into molecules which behave like hemolytically active C'1. Furthermore, actinomycin D or puromycin under appropriate conditions inhibits synthesis of C'1, suggesting that the increase in C'1 activity is the result of synthesis, not release of preformed C'1. The kinetics of inhibition by actinomycin D are in agreement with evidence that actinomycin probably blocks synthesis of mRNA. The time following the addition of actinomycin D during which there is nearly normal synthesis of C'1 may correspond to the time required to exhaust the supply of specific mRNA. Puromycin is an efficient inhibitor of C'1 production in a nutrient-poor medium (RL buffer), but incubation of intestinal tissue in an enriched medium at 30°C in the presence of puromycin results in an apparent enhancement of C'1 synthesis.

Summary.—Evidence is presented for the *in vitro* synthesis of C'1 by guinea pig small intestine and for the *in vitro* incorporation of C^{14}-lysine into hemolytically active C'1. Synthesis of C'1 by spleen, liver, kidney, lung, testes, esophagus, stomach, cecum, or colon could not be demonstrated.

Actinomycin D and puromycin inhibit C'1 synthesis by guinea pig small bowel and inhibition can be reversed by returning the tissue to media lacking inhibitors of protein synthesis. In an enriched medium, there is an apparent enhancement of C'1 synthesis in the presence of puromycin.

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[1] Yarmolinsky, M. B., and G. L. de la Haba, these PROCEEDINGS, **45**, 1721 (1959).

[2] Levinthal, C., A. Keynan, and A. Higa, these PROCEEDINGS, **48**, 1631 (1962).

[3] Mayer, M. M., in *Kabat and Mayer's Experimental Immunochemistry* (Springfield: Charles C Thomas, 1961), 2nd ed.

[4] Borsos, T., and H. J. Rapp, *J. Immunol.*, **91**, 851 (1963).

[5] *Ibid.*, **95**, 559 (1965).

[6] Becker, E. L., *J. Immunol.*, **84**, 299 (1960).

[7] Rapp, H. J., and T. Borsos, *J. Immunol.*, **91**, 826 (1963).

[8] Thorbecke, G. J., G. M. Hochwald, R. Van Furth, H. J. Muller-Eberhard, and E. B. Jacobson, in *Ciba Foundation Symposium on Complement*, ed. G. E. W. Wolstenholme and J. Knight (London: J. & A. Churchill, Ltd., 1965), p. 99.

[9] Hochwald, G. M., G. J. Thorbecke, and R. Asofsky, *J. Exptl. Med.*, **114**, 459 (1961).

[10] Siboo, R., and S. I. Vas, *Can. J. Microbiol.*, **11**, 415 (1965).