that of mammalian sera, and the usual terminology seemed valid under the experimental conditions outlined above.

Previous work with amphibians(7) and studies in progress on teleost fishes have shown that these groups generally possess lower levels of complement than the nurse and lemon sharks. Present evidence indicates, therefore, that no orderly decrease in complement levels occurs as the phylogenetic scale is descended through the *Elasmobranchii.*

Summary. Sera from 2 species of sharks exhibited a high degree of hemolytic activity for erythrocytes of several foreign species. Natural antibody was absorbed by trypsinized erythrocyte stromata and replaced by shark or turtle antibody. Potentiation by heterologous sensitizers and inactivation by heat, hydrazine, carrageenin, and EDTA suggested that these primitive vertebrates possess C′ systems similar to those of mammals. Quantitative C′ titers were comparable to those of guinea pig sera. Sting ray sera displayed extremely low levels of hemolytic activity which was not potentiated by heterologous sensitizers. Responses to inactivation procedures indicated that this activity might also be C′ mediated. An extreme lability to freezing was characteristic of elasmobranch sera.

Results suggested that no orderly decrease in C′ forming ability occurs as the phylogenetic scale is descended through the elasmobranch level, as has been suggested for other parameters of the immune response.

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Differentiation of Trachoma Bedsoniae *in vitro.** (31660)

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Immunologic typing of trachoma-inclusion conjuctivitis (TRIC) bedsoniae has been accomplished by immunizing mice followed by lethal toxic challenge (1,2,3,) or by immunofluorescence using absorbed human serum(4). Both of these tests have proven impractical for general use, the former because it is labo-

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TABLE I. TRIC Strains Used in Typing by Immunofluorescence.

Abbreviation	Official nomenclature[9]	Source
SA-1*	TRIC/ /SAU/HAR-1/OT	Murray *et al*(10)
SA-2†	TRIC/ /SAU/HAR-2/OT	*Idem*
SA-5*	TRIC/ /SAU/HAR-5/OC	"
SA-13†	TRIC/ /SAU/HAR-16/OT	"
Eg-2*	TRIC/ /ET/HAR-13/OT	"
HAR-31‡	TRIC/ /SAU/HAR-31/OT	R. L. Nichols, Dhahran Saudi Arabia
HAR-32‡	TRIC/ /SAU/HAR-32/OT	*Idem*
HAR-33‡	TRIC/ /SAU/HAR-33/OT	"
HAR-34‡	TRIC/ /SAU/HAR-34/OT	"
A-7†	TRIC/ /ETH/HAR-20/OT	Murray *et al*(11)
P-2*	TRIC/ /P/73-60,2/OT	A. Sampaio & L. Ayres(12)
TW-5	TRIC/ /Taiwan/NAMRU No. 2-5/OT	J. T. Grayston(13)
ND-3	TRIC/ /New Delhi/NAMRU No. 2-3/OT	*Idem*
LT-1†	TRIC/ /GB/MRC-7/OT	L. H. Collier(14)
G-1	TRIC/ /WAG/MRC-1/OT	*Idem*(15)

* Previously typed as type 1 in mouse protection tests(2).
† Previously typed as type 2 in mouse protection tests(2).
‡ In mouse protection tests HAR-31 typed as type 1, HAR-33 questionably as type 2, HAR-34 as both types 1 and 2 and HAR-32 was untypable as either 1 or 2.

rious and expensive, the latter because of the scarcity of suitable human serum. Several methods of differentiating broadly among the bedsoniae have been described(5,6,7); none of these has been proved acceptable for easy differentiation among TRIC types, although one report(6) indicated that two strains of trachoma, BOUR and ASGH, could be separated from each other. Virtually all attempts to classify bedsoniae have utilized what amounted to hyperimmune serum. This report will describe the type-specific effect of antiserum drawn 7-10 days after a single intracardiac inoculation of antigen and its application as a new, rapid test for typing TRIC bedsoniae.

Materials and methods. Antigens: Antigens for the preparation of antisera were prepared by considerable modification of that described by Fraser and Berman(7). All procedures were carried out at 4°C except where noted, and as nearly as possible under conditions of bacteriologic sterility. 0.2 ml of crude, highly infectious 50% yolk sac was inoculated into the allantoic cavity of chick embryos in the 11th day of incubation. Four days later allantoic fluids were harvested; these usually contained large numbers of elementary bodies although the infectious titer was low. 400-800 ml of allantoic fluid were centrifuged at 17,000 rpm in a Spinco Model L, rotor 21, for 45 minutes and the sediment blended briefly in one half original volume of 1% trypsin in 0.1 M phosphate buffer, pH 7.4. After 30 minutes incubation at 37°C, the material was recentrifuged at 17,000 rpm, the sediment again taken up in one half volume of buffer, blended briefly, and added to Celite, 1 g per 100 ml. This suspension was shaken thoroughly, allowed to stand 15 minutes and then centrifuged in a horizontal head in an International PR-2 centrifuge at 1300 rpm for 15 minutes. The supernate was recentrifuged at 17,000 rpm and the resulting sediment, carefully measured with a syringe and needle, taken up in 16 times its volume of phosphate buffer. To this was added 4 times its volume of Genetron 113 (trifluorotrichloroethane, Allied Chemical Corp. Morristown, N. J.), the suspension blended at high speed for 5 minutes, then centrifuged at 1000 rpm in a horizontal head, for 15 minutes. The slightly opalescent white aqueous phase was replaced with an equal volume of buffer and the blending and centrifuging repeated. The two aqueous' were combined and the remaining Genetron removed by evacuation with a vacuum pump for 30-60 minutes. Finally, this suspension was centrifuged at 17,000 rpm for 45 minutes and the white sediment, consisting almost entirely of pure elementary bodies, was resuspended with thorough mixing in 1/100th volume of original allantoic fluid in 0.2% formalin in phosphate

TABLE II. Reciprocals of Fluorescent Antibody Titers of Guinea Pig Antisera Tested Against 15 TRIC Antigens.*

	Antiserum														
	Type 1								Type 2						Untyped
Antigen	Eg-2	HAR-34	SA-5	P-2	SA-1	HAR-31	ND-3	HAR-32	SA-2	SA-13	HAR-33	LT-1	TW-5	A-7	G-1
Eg-2	*160*	160	80	80	80	160	40	320	10	40	40	10	0	20	5
HAR-34	160	*320*	160	320	640	160	80	640	160	40	5	5	10	10	10
SA-5	40	160	*80*	40	20	20	160	640	5	10	40	0	10	20	5
P-2	80	320	80	*320*	640	80	80	320	10	40	10	10	10	40	5
SA-1	80	320	160	640	*80*	160	40	320	10	20	10	0	0	20	5
HAR-31	320	320	160	640	80	*320*	80	160	10	40	0	0	0	80	10
ND-3	80	160	80	160	5	0	*160*	640	5	10	0	0	5	20	10
HAR-32	80	160	80	40	0	20	80	*640*	5	40	5	5	5	20	0
SA-2	0	40	0	10	0	0	5	5	*160*	160	5	0	40	160	0
SA-13	5	40	5	10	0	5	5	5	640	*160*	40	40	40	20	0
HAR-33	5	20	0	0	0	5	10	0	160	160	*40*	40	40	20	10
LT-1	10	20	0	10	0	0	10	0	160	160	20	*80*	40	160	5
TW-5	0	10	0	0	0	0	0	0	80	160	0	0	*40*	5	0
A-7	10	40	5	10	0	10	20	5	160	160	20	0	20	*160*	10
G-1	40	40	10	0	5	10	10	160	20	40	0	0	0	160	*80*

* 0=negative at 1:5.

TABLE III. Specificity Differences (S.P.D.)* Among 15 TRIC Strains.

	Antiserum														
	Type 1								Type 2						Untyped
Antigen	Eg-2	HAR-34	SA-5	P-2	SA-1	HAR-31	ND-3	HAR-32	SA-2	SA-13	HAR-33	LT-1	TW-5	A-7	G-1
Eg-2	–	1	2	3	1	0	3	2	10	7	5	7	10	7	6
HAR-34	1	–	0	0	0	1	2	1	3	5	7	8	7	7	6
SA-5	2	1	–	3	1	3	0	0	11	8	5	10	7	7	7
P-2	3	0	3	–	—4	1	2	4	9	7	9	8	9	7	11
SA-1	1	0	1	—4	–	1	6	6	8	8	7	10	9	8	8
HAR-31	0	1	3	1	1	–	8	6	11	8	10	12	11	6	8
ND-3	3	2	0	2	6	8	–	1	10	9	8	9	9	6	7
HAR-32	2	1	0	4	6	6	1	–	12	9	11	12	11	10	8
SA-2	10	3	11	9	8	11	10	12	–	—2	3	5	1	0	8
SA-13	7	5	8	7	8	8	9	9	—2	–	0	1	0	3	7
HAR-33	5	7	5	9	7	10	8	11	3	0	–	2	4	4	7
LT-1	7	8	10	8	10	12	9	12	5	1	2	–	5	5	9
TW-5	10	7	7	9	9	11	9	11	1	0	4	5	–	6	9
A-7	7	7	7	7	8	6	6	10	0	3	4	5	6	–	3
G-1	6	6	7	11	8	8	7	8	8	7	7	9	9	3	–

* See text.

buffered saline (PBS), pH 7.2.

Antisera: Guinea pigs raised in isolation and carefully observed for possible infection with bedsonia, particularly the GPIC agent(8), were bled from the heart and through the same needle administered 1.0 ml of the above antigen, diluted 1:5 in buffered saline. Seven to ten days later the guinea pigs were bled out and the sera of 4 animals pooled. More that 150 tests on individual animals indicated that there was little variation from animal to animal in their response to this intracardiac administration of a particular antigen. Pre-bleedings were invariably negative and the above 1:5 dilution of concentrated antigen was arbitrary, since titrations indicated that the material could be used undiluted or diluted as much as 1:5000.

Immunofluorescence: The techniques of the immunofluorescent test have been described (16,4). Antigens consisting of 5% crude yolk sac, diluted in PBS, were ordinarily kept at —60°C. It was noted, particularly with type 2 antigens, that this temperature appeared necessary, since unfixed slides stored at —20°C seemed to lose their staining qualities with time; in general, those antigens characterized as type 1 were easier to read and gave sharper results than type 2 antigens. Guinea pig antisera previously mixed with 50% crude normal yolk sac and PBS in a ratio of 1:2:2 to give a final starting dilution of 1:5, were incubated at 37°C for 1 hour and overnight at 4°C. They were then diluted 2-fold in PBS and overlaid onto acetone fixed antigens for 30 minutes at 37°C in a moist chamber. Following 2 washes for a total of 10 minutes, the plaques were overlaid with rabbit anti-guinea pig globulin conjugate at 37°C for 30 minutes, and this was then gently rinsed off. Slides were read by one of the authors (DMcC) without knowledge of the antigens involved and graded from 0 to 4 on the basis of brightness. A reading of 1 was definite typical fluorescence. Normal yolk sac control antigens were used in each immunofluorescent test with uniformly negative results, as were controls using conjugate and antigen alone.

TRIC Strains: 15 trachoma strains were used in reciprocal immunofluorescent tests. These are listed in Table I.

Results. Table II depicts the results of 15 reciprocal serum titrations on the 15 antigens. Table III shows these results in numerical form using a value of 0 for negative at 1:5, 1 for a titer of 1:5, 2 for 1:10, etc. according to Fraser and Berman's(7) modification of Magnuson's(17) method for calculating specificity differences (S.P.D.) between 2 strains:

$$\text{S.P.D.} = (A_a - A_b) + (B_b - B_a)$$

where A_a and B_b are the homologous scores for antisera A and B with antigens a and b, while A_b and B_a are the heterologous scores. Thus, a value of S.P.D. near 0 indicates identity or close relationship between two strains and the higher the value of the S.P.D., the more distinct the two strains in question. For example, the titer of Eg-2 antiserum *versus* itself is 1:160, a value of 6, and *versus* HAR-34 antigen it is also 1:160. The titer of HAR-34 antiserum *versus* itself is 1:320, a value of 7, and *versus* Eg-2 antigen is 1:160 [(6 — 6) + (7 — 6) = 1]. It is obvious that 8 of the 15 strains fall into one type, called here type 1. If one considers a score of 4 or below to be the dividing line for homogeneity, 4 strains, Eg-2, HAR-34, SA-5 and P-2 cross with all 8 strains. Strains SA-1 and HAR-31 cross with themselves but not with strains ND-3 and HAR-32 which also cross between themselves. Thus, these two pairs seem to form subtypes within type 1. These results are completely compatible with mouse protection tests previously performed in this laboratory(2).

Six other strains, called here type 2, are clearly distinct from type 1 strains. The solitary exception might be strain HAR-34 which seems to have antigenic characteristics of both types. This was also shown to be true in mouse protection tests in this laboratory. The type 2 strains in general were more difficult to type both on the basis of immunofluorescent staining and in their ability to elicit high antibody titers in guinea pigs, particularly strains LT-1 and HAR-33. As previously found in mouse protection tests(2) and FA tests using absorbed human serum (4), strain G-1 did not fall into either of these two distinct types.

An example of the rapidity with which this

typing test could be used occurred when strain SA-3(TRIC/ /SAU/HAR-3/OT) was reisolated from the original specimen 8 years after its first isolation and 10 years after collection. This conjunctival eye scraping had been stored at —60°C diluted in sucrose-PG(18). The reisolate was recovered after 2 passages in yolk sac and typed the day of recovery. Using 5% dilute yolk sac as antigen, both the original strain and the reisolate reacted in high titer against three type 1 guinea pig antisera (Eg-2, HAR-31 and HAR-32) and in low titer, if at all, against two type 2 antisera (SA-2 and SA-13).

Discussion. To our knowledge all serologic investigation of TRIC bedsonia have utilized what amounted to hyperimmune sera, presumably containing mainly 7S gamma globulins. The antisera produced in 7-10 days by a single intracardiac inoculation is probably 19S gamma globulin, since in a limited number of complement fixation tests, titers were markedly lowered by treatment of the sera with ethanethiol. Furthermore, while 3 lots of antiguinea pig conjugate prepared in rabbits with crude gamma globulin and one lot with mainly 19S gamma globulin proved satisfactory, a preparation containing mainly anti-7S gamma globulin either failed to stain or gave minimal unsatisfactory staining in the above described systems. There is good reason to believe that the single massive intracardiac inoculation of highly purified antigen results in antiserum which is type specific. Multiple inoculation of either rabbits or guinea pigs in this laboratory has invariably resulted in loss of this specificity, although high titer group antisera can be produced. Hanna and Bernkopf(6) immunized rabbits with multiple subcutaneous or intramuscular inoculations over a period of weeks. They stated that those receiving the least intensive immunization showed "lower antibody titer but an apparent higher specificity" and they were able to distinguish between strains BOUR and ASGH. It is believed that the use of earlier antisera following a single intracardiac inoculation results in more specific antisera, which can be used to type TRIC bedsonia.

Correlation between the typing of TRIC bedsonia *in vitro* by the above method and by the mouse protection tests done in this laboratory is excellent. All results indicate that the two typing methods are measuring the same immunologic phenomenon. The mouse protection test has been too time-consuming, expensive and cumbersome for routine use. With the above described serologic test, it should be possible to type TRIC bedsonia and separate TRIC from other bedsonia with elimination of the disadvantages of the mouse test.

Summary. A rapid, specific immunfluorescent test for typing TRIC bedsonia is described. Antisera are prepared by inoculating highly purified antigen intracardially in guinea pigs which are bled in 7-10 days. This *in vitro* test correlates closely with mouse protection tests and the results indicate that there are at least 3 immunologic types of TRIC bedsonia.

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Biosynthesis of Thiamine. Inhibition of Thiamine Phosphatase Activity by Pyridoxal. (31661)

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Growth inhibition of *Neurospora crassa* cultures(1) grown in the presence of pyridoxal have been investigated and related to inhibitory effects on thiamine biosynthesis(2,3). The availability of cell-free preparations of yeast which biosynthesize readily measurable quantities of thiamine(4) from its precursors (hydroxymethylpyrimidine and thiazole)* has allowed several groups to establish the following sequence of reactions(5):

1) hydroxymethylpyrimidine —ATP; hydroxymethylpyrimidine kinase→ hydroxymethylpyrimidine-P

2) hydroxymethylpyrimidine-P —ATP; hydroxymethylpyrimidine phosphokinase→ hydroxymethylpyrimidine-PP

3) thiazole —ATP; thiazole kinase→ thiazole-P

4) hydroxymethylpyrimidine-PP + thiazole-P —thiamine-P synthase→ thiamine-P + PPi

5) thiamine-P —thiamine phosphatase→ thiamine + Pi

Using this yeast cell-free system it has been shown that pyridoxal-P and pyridoxal inhibit thiamine formation, although the other pyridoximers (pyridoxamine, pyridoxamine-P, and pyridoxine) do not(6). Evidence was presented to indicate that the inhibition caused by pyridoxal-P occurred at (or after) reaction 4 of the sequence(6). This paper continues the investigation of the inhibition caused by pyridoxal.

Materials and methods. Samples of hydroxymethylpyrimidine, the corresponding bromomethyl derivative, and thiazole were generously supplied by Merck and Co., Inc. Thiamine hydrochloride was obtained from Eastman Distillation Products Industries; thiamine phosphate from Calbiochem; pyridoxal·HCl and ATP (disodium salt) from Sigma Chemical Corp. Thiazole-P was prepared from thiamine-P by the method of Camiener and Brown(7). Hydroxymethyl-P and hydroxymethyl-PP were prepared from bromomethylpyrimidine by the method of Lewin and Brown(6).

Preparation of incubation mixtures. Cell-free extracts of baker's yeast (Fleischman's) were prepared and purified by ammonium sulfate fractionation procedures, as described by Camiener and Brown(7). These enzyme preparations were used to prepare thiamine-biosynthesizing incubation mixtures, which were incubated with or without pyridoxal at 38°C for specified lengths of time and then were heat inactivated at 100°C for 5 minutes (7). Further details are provided in connection with the appropriate Figures and Tables.

Microbiological determination of thiamine. Aliquots of incubation mixtures were diluted and thiamine content determined by microbiological assay using *Lactobacillus viridescens* A.T.C.C. 12706, according to the meth-

* The following abbreviations are used in this paper: hydroxymethylpyrimidine for 2-methyl-4-amino-5-hydroxymethylpyrimidine; bromoethylpyrimidine for 2-methyl-4-amino-5-bromomethylpyrimidine; thiazole for 4-methyl-5-(2-hydroxyethyl)thiazole; ATP for adenosine triphosphate; P and PP for phosphate and pyrophosphate, respectively.