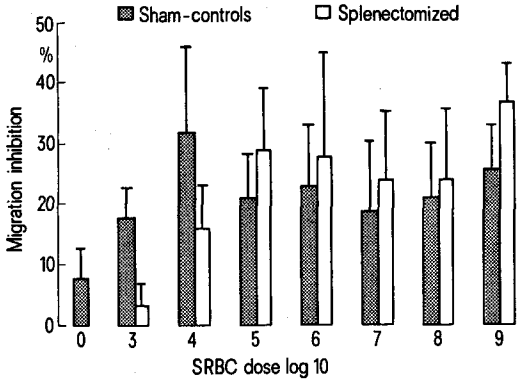
splenectomy created no dearth of competent lymphocytes that mediate MI while removing the major source of antibody production.

Materials and methods. Adult lizards were maintained[8] and splenectomized[5] as detailed elsewhere. Different dilutions of SRBC in phosphate-buffered saline (PBS) were given i.m. to both sham-operated and splenectomized lizards 7 days after surgery. 9 parts of blood in Alsever's solution was mixed with one part of 6% Dextran (mol wt 5,000,000; Sigma Chemical Co., USA) and kept at room temperature for 1.5 h to separate peripheral blood white cells of *Calotes*[9]. Peritoneal exudate cells (PEC) were obtained by washing the peritoneum with calcium-magnesium-free PBS (with antibiotics and 5.0 IU heparin/ml) 4 days after the i.p. injection of 4–6 ml of heavy paraffin oil[9]. 14 days after immunization, PFC[8] and MI assays were performed. The method for MI assay utilizing PEC was similar to that described for *Calotes* spleen cells[3]. After 24 h of incubation at 37 °C, the areas of migration of PEC cultured with and without antigen were compared and the degree of MI was calculated. Statistical assessment of data was by Student's t-test.

Effect of splenectomy on PFC response to SRBC*

Group	Spleen	Peripheral blood white cells	Peritoneal exudates
Unsensitized controls	8.5±4.4** (5)***	1.0±0.7** (5)***	14.2±5.4** (11)***
Sham-operated controls	210.5±31.8 (6)	107.3±20.4 (6)	46.0±22.0 (6)
Splenectomized	–	11.1±5.3 (5)	15.5±8.7 (5)

*PFC assay was performed 14 days after immunization. **Arithmatic mean ± SE. ***Number of animals investigated.



Effect of splenectomy on MI response to SRBC. Both sham-operated and splenectomized lizards were immunized with varying dilution of SRBC and assayed 14 days later for MI response. Each point represents the mean ± SE of 4–10 animals.

Results and discussion. Previous studies have shown that the intact spleen is an absolute necessity for *Calotes* to produce antibodies to i.p. injection of SRBC[5] or bovine serum albumin[7] and also to manifest anaphylaxis to an intracardiac injection of egg albumin[6]. In the present study, i.m. route was chosen to immunize the lizards since this route favours the efficient elicitation of both CMI and HI to SRBC[3,4]. Hence, an experiment was performed to ensure that splenectomy would erase the HI response irrespective of the route of injection of SRBC. PFC were monitored in peripheral blood and PEC, since it was already known that splenectomy abrogated the humoral antibody production[5,7]. I.m. injection of 10^8 SRBC failed to induce PFC generation in peripheral blood and PEC (table), thereby confirming the earlier reports[5–7]. The next experiment was conducted to analyse the status of CMI in animals that were deprived of HI by splenectomy. The data (figure) indicate that splenectomy did not impair the ability of lizards to mount CMI to SRBC doses ranging from 10^4 to 10^9. However, the lowest dose, 10^3 SRBC, failed to sensitize the splenectomized lizards ($p < 0.05$) for MI response.

The observation that splenectomy affected the CMI response to very low SRBC dose is in keeping with the findings in mice[11]. Similarly, in toads the antibody synthesis to SRBC and human gamma-globulin was affected by splenectomy only to low but not to high threshold antigen levels[12]. A plausible interpretation could be that the low antigen dose has minimal chances to interact with competent lymphocytes in the absence of spleen[11]. Splenectomized lizards did not show any increment in the level of MI to high doses of SRBC ($p > 0.5$), unlike mice[11]. Similar results were obtained with Cyclophosphamide and Freund's complete adjuvant treatment[9]. This evidence suggests the total independence of MI on HI in lizards.

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Temperature variation and the function of complement and antibody of amphibia[1]

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Summary. Temperature treatment of sera of erythrocyte immunized adults of 3 amphibian species has shown that cooling enhances antigen-antibody complexing and does not alter complement function in vitro. These findings bear on understanding in vivo temperature immunosuppression in poikilotherms.

Cohen[2] has recently discussed the consequence of large daily or seasonal environmental temperature fluctuations on immunity and pathogenesis in poikilotherms, placing particular emphasis on growth rates of bacteria and viruses as they affect reptiles. It is known that temperature depression will inhibit allograft rejection in fish[3,4] and amphibia[5–8], as well as humoral immunity of fish[9–11], amphibia[12–17] and reptiles[18]. It is conceivable then that different parts of the adaptive immune response may represent temperature sensitive events. It has recently

been reported[19,20] that dissociated splenocytes from immunized *Triturus viridescens*, the common American newt and the grass frog, *Rana pipiens*, can both synthesize and secrete antibody in vitro at 4 °C. Others have found that hibernation temperatures will depress the level of recoverable complement in the serum of unimmunized *Rana pipiens*[21]. The nature of complement in fish[22–27] and amphibia[28,29] had been studied earlier. At the other end of the temperature scale, Weinheimer et al.[29] reported that 48 °C was adequate for decomplementation of serum of the marine toad, *Bufo marinus*. Yet, investigators have continued to use 56 °C, the decomplementation temperature for mammalian sera, when studying amphibian antibody activity. However, Tochinai et al.[30], have found that decomplementation of South African clawed toad, *Xenopus laevis*, serum for 30 min at 56 °C reduces the level of antibody activity by 50–75% as measured by microtiter hemagglutinin assays. The experiments reported here were designed to determine the activity of amphibian complement and antibody after temperature treatment of immune sera at 10 °C intervals, from 5 °C on to 55 °C.

Methods. Adults of 3 species, *Triturus viridescens*, *Xenopus laevis* and *Rana pipiens*, were immunized by i.p. injection of 20% horse erythrocytes (HRBC) in Alsever's solution (Colorado Serum Co.). The newts received 0.2 ml, while larger individuals of the other 2 species received 0.5 ml of the challenge dose. Comparable levels of antibody activity were obtained by challenging *Triturus* 3 times, *Xenopus* twice and *Rana* once. Multiple injections were made at weekly intervals and blood was collected 10 days after the terminal injection. Blood was withdrawn from the heart with glass capillary tubing and allowed to clot for 1 h at room temperature and then for 4 h at 5 °C. The serum was separated from the clot by centrifugation and divided into 6 aliquots. Each of these was subjected to one of the various temperatures for 40 min. In those cases where sera were pretreated at 25 °C or higher, they were subsequently serially diluted at room temperature into 12 wells of a microtiter plate (Cooke Engineering), 25 µl of 1% HRBC was added to each well and the plates placed overnight at 25 °C before being observed. Sera treated at 5 °C or 15 °C were diluted and stored overnight at those low temperatures. All experiments were performed a minimum of 3 times with sera pooled from different groups of animals.

Complement-mediated lysis and hemagglutinin activities of temperature treated immune sera

	Pre-incubation temperature (°C)					
	5[a]	15	25	35	45	55
Rana						
Lysis	67±0[b]	67±0	100±0	100±0	30±24	0±0
Agglutination	150±0	150±0	100±0	100±0	88±3	13±12
Xenopus						
Lysis	78±26	83±28	100±0	94±8	4±7	0±0
Agglutination	128±25	123±20	100±0	100±0	100±0	91±7
Triturus						
Lysis	48±13	77±21	100±0	93±15	27±37	14±22
Agglutination	140±36	139±38	100±0	103±6	88±13	90±26

Activities resulting from the 25 °C treatment are set at 100, all other activities are expressed relative to this reference.

[a] Assays for lysis and agglutination for the 5 °C and 15 °C pretreated sera were performed at 5 °C and 15 °C respectively. All others were assayed at 25 °C. [b] From at least 3 determinations, mean ± SD.

Results and discussion. On the average, 6–8 wells of RBC specific antibody activity was obtained at 25 °C. The first 4–5 wells demonstrated hemolysis, while the remaining wells showed hemagglutination. Additional tests were made to determine if the lysis observed under these conditions could serve as a measure of complement activity. Lysis, but not agglutination, was blocked by 30 mM EDTA. In addition, the original number of wells of lysis could be restored to heat inactivated immune sera by the addition of HRBC-adsorbed nonimmune serum as a complement source. Our results showed that 45 °C for 40 min was adequate for complete decomplementation of sera from *Triturus* and *Xenopus*, while lytic activity of *Rana* serum was reduced by 80%. No substantial reduction of hemagglutination occurred after 55 °C treatment of *Triturus* and *Xenopus* sera. *Rana* serum, however, showed no lytic or hemagglutinin activity after 40 min at 55 °C. Temperatures of 5 °C and 15 °C actually increased the level of hemagglutinin activity in all 3 species by 25%, when compared to the same sera at 25 °C. No change in the level of complement activity (lysis) was seen. Additionally, we found that freezing and thawing through at least 6 cycles had no effect on either lytic or agglutinin activities. Unimmunized sera from

1 This research was partially supported by a grant No. AI-12846 from the National Institutes of Health, Bethesda, Md, USA. The authors are grateful for the technical assistance of Ms J. S. Ruben.

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individuals of these 3 species, completely adsorbed with sheep erythrocytes, were also tested for their ability to serve as a complement source for rabbit-anti-sheep hemolysin (GIBCO). All showed this capacity. However, when unimmunized rat serum was used as a complement source for rabbit-anti-sheep hemolysin, no lysis was obtained at 15 °C or lower. Legler et al.[28] have shown that *Bufo marinus*, *Necturus maclosus* and *Rana pipiens* complement can function with mammalian but not fish antibody.

Thus, complement and antibody of these poikilotherms seem best adapted to function at environmental temperatures in which these organisms normally live. While the laboratory temperature of 25 °C has been used in the past in an effort to provide optimal temperature conditions for these particular amphibia, our data suggest that it is not optimum for serum agglutination activity. Since our data show clearly that amphibian complement functions in vitro at the hibernation temperatures which have been found to reduce the amount of complement activity recoverable from *Rana* serum[21], it would seem that immune inhibition at cold temperatures may be partly due to a suppression of synthesis rather than activity of complement. Therefore previously activated amphibian immunocytes can continue to generate and release antibody in vitro[19,20], complement function is normal, although its synthesis in vivo is depressed[21] and antigen-antibody complexing is enhanced by the cold. If the effect of low temperature on the in vivo immune response has to do with threshold activity of a particular temperature sensitive phase, only the phenomena associated with activation of lymphocytes by antigen remain.

Changes in rate of methemoglobin reduction and oxygen affinity of erythrocytes incubated with inosine, pyruvate and phosphate

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Summary. Incubation of human erythrocytes with inosine, pyruvate and phosphate increases several fold the ferrihemoglobin reductase activity, the values of which, however, depend on the age of blood (by 6 to 2 times with respect to the normal value of fresh blood).

Blood stored in acid-citrate-dextrose (ACD) shows a progressive decrease in the activity of the ferrihemoglobin reduction systems and a concomitant increase in oxygen affinity[1,2]; these functional changes are related to the metabolism of the erythrocyte and to the exchange of materials between cells and preservation solution. Incubation at 37 °C of outdated whole blood or concentrated red cells in a medium containing inosine and adenine[3,4] or inosine, pyruvate and phosphate[5,6] restores the defective oxygen transport function and the depleted 2,3-diphosphoglycerate (DPG) concentration of erythrocytes. Moreover the incubation with rejuvenation solutions determines an increase in the 24-h post-transfusion survival[6,7]. Due to the possible practical utilization of this procedure, several studies have been carried out on biochemical modifications of erythrocyte metabolism after incubation with various substances[8–11]. The purpose of this comunication is to report the effect of inosine (10 mM), pyruvate and inorganic phosphate (5 mM) (IPP solution) on the activity of the ferrihemoglobin reduction system, the oxygen affinity and the osmotic fragility of stored erythrocytes. The techniques used have been described previously[2].

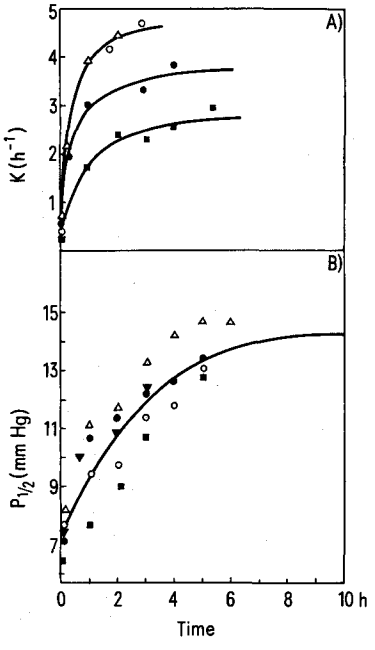

Fig. 1. Effect of incubation erythrocytes with inosine (10 mM), pyruvate (10 mM) and phosphate (5 mM) at 37 °C in isotonic Tris buffer pH 7.3. *A* Change in the rate of ferrihemoglobin reduction as a function of time of red cells stored at 4 °C for 2 h and 3, 16 and 30 days; *B* change in $p^1/_2$ (the oxygen tension at 50% oxygen saturation), measured at 20 °C in isotonic Tris buffer pH 7.3, of red cells. ▼, △, 2 days old; ○, 4 days old; ●, 16 days old, ■, 30 days old.

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