indicates that this species is susceptible to anaphylaxis, although it seems at present that this sensitivity is inferior to that observed in other laboratory animals. Additional studies will be needed for a more precise evaluation of the biological properties of cat anaphylactic antibodies.

BESREDKA[12] was the first to introduce an immuno-neurological model by using the intracerebral route in studying the nervous origin of anaphylactic symptoms. His approach to the investigation of immune apparatus and its manifestations still represents the most provocative subject of experimental medicine, and undoubtedly deserves to be explored by means of all the technical and philosophical potentials of contemporary immunology and neurosciences[13].

Résumé. La γ-globuline bovine, en injections intraveineuses et dans le ventricule latéral du cerveau, a produit, chez le chat, l'apparition d'anticorps spécifiques dans la circulation et la sensibilisation déterminant une réaction anaphylactique d'intensité variable.

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[12] A. BESREDKA, *Anaphylaxis and Anti-Anaphylaxis and Their Experimental Foundations* (C. V. Mosby, St. Louis 1919), p. 13.

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Effect of Bilirubin on Glucose Oxidation in Red Cells

There has been ample evidence that the erythrocytes bind unconjugated bilirubin[1,2]. Bilirubin combined with human erythrocytes may be a factor in the shortened red cell survivals observed in erythrocytes pre-incubated in the solutions of unconjugated bilirubin[3]. The purpose of the present study is to elucidate the effect of bilirubin on glucose oxidation in erythrocytes in view of the fact that glycolysis is the main source of the red cell's energy.

The blood used in the study was obtained from healthy adult volunteers. Venous blood was obtained from the subjects with use of heparin as an anticoagulant. The blood was centrifuged at 1500 rpm for 10 min and the plasma and buffy coat were removed by aspiration. The packed erythrocytes were not subjected to further washing by buffer solution to avoid lowering ATP levels[4].

Experiment 1. One part of the packed erythrocytes was mixed with 1 part of either one of the following solutions: Solution A containing bilirubin; bilirubin (Daiichi Chemical Co., Tokyo) was dissolved in 0.1 *N* Na_2CO_3 solution at a concentration of 1.0%[5]. The bilirubin solution was then mixed with Krebs-Ringer phosphate buffer containing 1% human albumin (Sigma Chemical Company) to give a final concentration of bilirubin of 0.03%. The solution was freshly prepared and the pH was adjusted to 7.4 with 1 *N* HCl.

Solution B: Prepared as described above except that bilirubin was omitted. Each of the red cell suspensions thus prepared was divided into 2 equal portions. The first portion was incubated in the dark with occasional gentle agitations at 37 °C for 50 min, aiming at facilitating the binding of bilirubin with the red cells[1,2]. After the incubation, the red cells were resuspended without washing in Krebs-Ringer phosphate buffer (pH 7.4) to give a hematocrit reading of 0.40–0.50. The second portion of the red cell suspension was not incubated as described above.

$^8/_{10}$ ml of 1 of the red cell suspensions and 0.1 ml of ^{14}C-U-glucose[6] (125,000 dpm) containing 16 mg glucose were placed in a 25 ml flask equipped with a center well. A rubber stopper was attached to the flask. 1 ml of $^{14}CO_2$ trapping solution (1 part ethanolamine and 2 parts ethylene glycol)[7] was injected through the rubber stopper of the flask into the center well. These aliquots were incubated on a metabolic shaker and shaken at 45–50

[1] D. WATSON, Clin. chim. Acta *7*, 733 (1962).
[2] A. F. OSKI and J. L. NAIMAN, J. Pediat. *63*, 1034 (1963).
[3] A. SAWITSKY, E. SEIFTER and S. BRAMSON, Proc. Int. Congr. on Hematology, Mexico City, September 1962.
[4] F. A. OSKI and J. L. NAIMAN, Pediatrics *36*, 104 (1965).
[5] B. H. BILLING, R. WILLIAMS and T. G. RICHARDS, Clin. Sci. *27*, 245 (1964).
[6] ^{14}C-U-glucose designates a solution in which the glucose was labelled with carbon equally in all positions.
[7] D. Y. HSIA and T. INOUYE, in *Inborn Errors of Metabolism* (Year Book Medical Publishers, Chicago 1966), Part 2, p. 11.

Effect of bilirubin and methylene blue on the glycolytic activities of the erythrocytes

	Experiment 1					Experiment 2				
	Erythrocytes		Erythrocytes; Bilirubin		*t*-test	Erythrocytes; Methylene blue		Erythrocytes; Methylene blue; Bilirubin		*t*-test
	$n = 6$ Mean	S.D.	$n = 6$ Mean	S.D.		$n = 6$ Mean	S.D.	$n = 6$ Mean	S.D.	
No preincubation	0.169	0.039	0.154	0.020	$p > 0.05$	3.37	0.43	2.25	0.14	$p < 0.01$
With preincubation	0.141	0.012	0.145	0.025	$p > 0.05$	3.82	0.50	2.25	0.25	$p < 0.01$

The glycolytic activities of the erythrocytes are expressed as percent of the initial radioactivities of the ^{14}C-U-glucose released as $^{14}CO_2$.

cycles/min at 37 °C for 2 h. $^2/_{10}$ ml of 1 N H_2SO_4 was injected into the aliquot after the 2-h-incubation period. An additional 20 min incubation followed and then the $^{14}CO_2$ was transferred to a vial which contained 10 ml of scintillation fluid (2.5-diphenyloxazole, 4 g and 1.4-*bis*-2-(5 phenyloxazolyl) benzene 0.1 g/l of toluene). The $^{14}CO_2$ was counted in an Aloka liquid scintillation counter (Nihon Musen Co., Tokyo) and the dpm were computed from the measured efficiency of the counter. The percent of the initial radioactivity of the ^{14}C-U-glucose released as $^{14}CO_2$ was calculated.

Experiment 2. The red cell suspensions, solutions A and B, were prepared and mixed and incubated as mentioned in experiment 1. The red cell suspensions, ^{14}C-U-glucose solution, and 0.1 ml of 0.05% methylene blue solution were placed in the flask. The methylene blue was added in an effort to activate glucose monophosphate shunt in the erythrocyte. It has been known that methylene blue stimulates the mechanism of oxydation of hexose-6-phosphate in the erythrocyte by carrying electrons directly to oxygen, thereby permitting the glucose oxidation pathway to recycle[5]. Processes afterwards were the same as those in experiment 1.

The results of the experiments are presented in the Table. As can be seen from the Table, the glycolytic activities of the erythrocytes, expressed as the rates of production of $^{14}CO_2$ from ^{14}C-U-glucose, did not alter in the presence of bilirubin either when the erythrocyte-bilirubin mixture was preincubated ($p > 0.05$) or when it was not preincubated ($p > 0.05$). Since the results were against the expectation that bilirubin may interfere with the glycolytic activities of the erythrocytes, it was felt that further study in the presence of methylene blue would be necessary before offering an explanation for the results. The addition of methylene blue resulted in the increase of glycolytic activities of the erythrocytes by approximately 20 times, confirming the earlier work of BRIN and YONEMOTO[8]. When bilirubin was added to the erythrocyte-methylene blue system, however, the increase of glycolytic activities was markedly impaired. The difference between the 2 was statistically significant both when erythrocyte-bilirubin mixture was preincubated ($p < 0.01$) and when it was not preincubated ($p < 0.01$). Methylene blue has been known to increase the activities of hexose monophosphate shunt pathway in the erythrocytes[8]. Probably an increased reoxidation of the NADPH in the presence of methylene blue will regenerate the reactions of this pathway[9]. Our findings, therefore, suggest that bilirubin might interfere with the transfer of electrons from NADPH to methylene blue and in turn depress the glycolytic activities of the erythrocytes. This interference of bilirubin may be a factor in the recently observed shortened red cell survivals noted in erythrocytes preincubated in the solution containing unconjugated bilirubin[3].

Zusammenfassung. Es wird nachgewiesen, dass die $^{14}CO_2$-Herstellung aus ^{14}C-U-Glukose durch rote Blutkörperchen in Anwesenheit von Methylenblau sich um das etwa 20fache vermehrt. Der Einfluss von Methylenblau wurde auffallend herabgesetzt, wenn Bilirubin dem Erythrozytensystem mit Methylenblau zugesetzt wurde.

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Department of Pediatrics,
Hokkaido University, School of Medicine,
Sapporo (Japan), 31 March 1969.

[8] M. BRIN and R. H. YONEMOTO, J. biol. Chem. *230*, 308 (1958).
[9] A. SZEINBERG and P. A. MARKS, J. clin. Invest. *40*, 914 (1961).

Increased Primary Immune Response and Priming in Mice to Subimmunogenic Doses of Sheep Erythrocytes by *Bordetella pertussis*

The injection of killed cells of *Bordetella pertussis* into mice, alone or together with a second antigen, results in a long-term increase of the wet and dry spleen weights, characterized by a doubling of the cell number and an increased protein synthesis of the individual cell[1]. In this multiplication, besides other proliferating cell systems, antibody – forming cells participate[2,3]. The present study was performed in order to investigate, at the cellular and humoral level, whether the primary immune reaction and the process of priming for the secondary response are altered by *B. pertussis*, if subimmunogenic doses of sheep erythrocytes are used as an antigenic stimulus.

Adult male mice of the inbred strain NMRI weighing 19–26 g were immunized i.p. with 4×10^6 sheep erythrocytes (SE) (group I). In preliminary studies this dose had been determined as the threshold dose allowing the development of a measurable immune response at the cellular level. A second group of mice (group II) was immunized by the simultaneous i.p. injection of 4×10^6 SE and 3×10^9 cells of *B. pertussis* (phase I, not absorbed and killed by treatment with merthiolate for 30 min at 60 °C). A second i.p. injection of 4×10^6 SE was given into the mice of both groups 37 days after the primary antigenic stimulus. At different intervals after the primary and secondary immunization, 5 mice out of each group and 2 animals of the corresponding controls were

The influence of *Bordetella pertussis* (BP) on the development of direct and indirect plaque-forming spleen-cells to a subimmunogenic dose of sheep erythrocytes (SE) in mice

Days after primary immunization	Average numbers of PFC/10^6 spleen cells in mice immunized with 4×10^6 SE		4×10^6 SE + 3×10^9 BP	
	Direct PFC	Indirect PFC	Direct PFC	Indirect PFC
2	1	0	17	0
3	1	0	103	0
4	10	0	122	0
5	31	8	382	509
7	3	0	414	314
10	8	27	169	781
13	2	2	97	939
37	3	3	24	145
40	1	1	26	164
42	4	4	17	82
44	5	0	31	208

[1] H. FINGER, G. BENEKE and P. EMMERLING, Z. med. mikrobiol. Immunol. *154*, 23 (1968).
[2] H. FINGER, P. EMMERLING and H. SCHMIDT, Experientia *23*, 591 (1967).
[3] H. FINGER, P. EMMERLING, H. TUSCH and W. BREDT, Z. ImmunForsch. exp. Ther. *136*, 268 (1968).