monal component signalling aggregation and host colonisation. The obvious dependence of (4S)-cis-verbenol production on the chemical makeup of a host tree's oleoresin, and particularly the enantiomeric composition of the α-pinene contained therein, suggests a unique feed-back system in the plant/insect relationship. Potentially, a host tree supports the insect pest's aggregation system to the extent to which the tree's oleoresin contains (−)-α-pinene. Besides such ecological implications, the experimental procedure described may serve as a simple method in the gross examination of resinous materials as to their enantiomeric composition of α-pinene.

1 Supported by Stiftung Volkswagenwerk and Deutsche Forschungsgemeinschaft. We thank Prof. J.P. Vité for helpful suggestions and critical review of the manuscript and Dr J. Lulev for providing oleoresin samples.
2 J.P. Vité and W. Francke, Naturwissenschaften *63*, 550 (1976).
3 J.P. Vité, A. Bakke and A.A. Renwick, Can. Ent. *104*, 1967 (1972).
4 P.R. Hughes, J. Insect Physiol. *20*, 1271 (1974).
5 J.A. Byers, D.L. Wood, L.E. Brown, B. Fish, B. Piatek and L.B. Hendry, J. Insect Physiol. *25*, 477 (1979).
6 A.A. Renwick, P.R. Hughes and J.S. Krull, Science *191*, 199 (1976).
7 E. Hackstein and J.P. Vité, Mitt. dtsch. Ges. ang. allg. Ent. *1*, 185 (1978).
8 J.M. Brand, J.W. Bracke, A.J. Markovetz, D.L. Wood and L.E. Brown, Nature *254*, 136 (1975).
9 A.E. Comyns and H.J. Lucas, J. Am. chem. Soc. *79*, 4339 (1957).
10 J. Lulev, U. Brümmer and W. Francke, Allg. Forst- u. Jagdztg. *149*, 173 (1978).
11 K. Kruse, W. Francke and W.A. König, J. Chromat. *170*, 423 (1979); W. Francke, P. Sauerwein, J.P. Vité and D. Klimetzek, Naturwissenschaften *67*, 147 (1980).
12 N. Sakota and S. Tanaka, Bull. chem. Soc. Jap. *44*, 485 (1971).
13 J.A.A. Renwick, P.R. Hughes and J.T. De Ty, J. Insect Physiol. *19*, 1735 (1973); L.M. Libbey, M.E. Morgan, T.B. Putnam and J.A. Rudinsky, J. Insect Physiol. *20*, 1667 (1974).

Reduction of ferricytochrome c by human red cells

A. Tomoda, M. Ida, Y. Yoneyama, S. Kitajima and S. Minakami

Department of Biochemistry, Kanazawa University, School of Medicine, Kanazawa 920 (Japan), and Department of Biochemistry, Faculty of Medicine, Kyushu University, Fukuoka 813 (Japan), 26 February 1980

Summary. Human red cells reduced extracellular ferricytochrome c to ferrocytochrome c under various conditions, suggesting that ferricytochrome c reducing systems are present at the outer surface of the red cell membrane.

Though it has been indicated that there are some electron transfer systems in the human red cell membrane, little is known about their nature. Mishra and Passow[1] observed that the ferricyanide anion, which does not penetrate into red cells, is reduced by human red cells. They considered that this phenomenon is probably correlated with intracellular metabolism, because the reduction of ferricyanide decreased under conditions in which red cell glycolysis is depressed. Recently Orringer and Roer[2] proposed that ferricyanide may be reduced by ascorbate-mediated transmembrane reducing systems of the cell membrane. In spite of these reports, the possibility that the reducing systems are present in the outer membrane of the red cells is not discussed.

In the work described in this paper, we investigated whether or not ferricytochrome c, which also does not penetrate into red cells (own unpublished data), can be reduced by whole human red cells. We found that ferricytochrome c was reduced significantly by human red cells, and that the reduction was correlated neither with intracellular metabolism nor with ascrobate-mediated transmembrane reducing systems of the cell membrane. Our results suggested that ferricytochrome c reducing systems are present on the outer surface of the red cell membrane.

Methods. Red cells were obtained from 1-day-old ACD blood after centrifugation at 3000 rev./min for 10 min. After removal of buffy coats and plasma, the red cells were washed with about 5 vol. of 0.9% NaCl solution 5 times. By this procedure, leucocytes and thrombocytes were almost completely removed, and glucose-free red cells were obtained. Then the red cells were suspended in Krebs-Ringer solution containing 120 mM NaCl, 2 mM potassium phosphate, 1 mM $MgCl_2$, 5 mM KCl (hematocrit value; 30%). The pH of the suspension was adjusted to 7.4 by the addition of 0.1 M NaOH in Krebs-Ringer solution at 37 °C. The red cell suspensions were used for the following experiments. 1. Either NADH, NADPH (final concentrations; 1 mM), NAD, NADP (5 mM), FMN (1 mM), superoxide dismutase (116 units) or ascorbate oxidase (14.7 units) was added to 25 ml of red cell suspensions, which were incubated at 37 °C after the addition of glucose (10 mM). The reaction was initiated by the addition of ferricytochrome c solutions (200 μM). 5 ml of the samples were taken out at intervals for analysis, and centrifuged immediately at 10,000 rev./min for 1 min. There was no hemolysis due to these treatments. The supernatants were diluted with 4 vol. of Krebs-Ringer solution, and the absorption spectra of the supernatants were measured between 450 and 650 nm spectrophotometrically. 2. The experimental conditions are the same as those stated in (1), except that 1 mM deoxyglucose was added in place of glucose. 3. 2 mM NEM or PCMBS was added to the red cell suspensions, which were further held at 37 °C for 5 min. Free NEM and PCMBS were removed from the suspension by washing with Krebs-Ringer solutions (centrifuged at 8000 rev./min for 1 min twice). The red cells obtained by this procedure were resuspended in a Krebs-Ringer solution to make at 30% hematocrit. After adjustment of the pH of the suspension to 7.4 with 0.1 M NaOH dissolved in Krebs-Ringer solution, the reaction was initiated by the addition of ferricytochrome c solution. The absorption spectrum of the supernatant was measured.

Results. Figure 1, A shows the absorption spectra of the ferricytochrome c containing supernatants of the samples at 0-90 min (control) between 450 and 650 nm. The absorbance at 521 and 550 nm increased with time, and the isosbestic points were clearly observed at 502, 526, 542 and 556 nm. The addition of NADH, NADPH, NAD, NADP,

FMN, superoxide dismutase, ascorbate oxidase, and deoxyglucose, however, had no effect on the reduction of ferricytochrome c by the cells. Figure 1, B shows the time-course plots of the optical density of the supernatant at 550 nm. Deoxyglucose and ascrobate oxidase produced no alteration in the rate of ferricytochrome c reduction.

Further, the effects of red cell concentration on the reduction rate of ferricytochrome c were studied. As shown in figure 2, the first-order reaction rate constants, which were estimated from the increase in absorbance at 550 nm, showed a linear relationship with red cell concentrations (hematocrit values). This result suggests that the reduction of ferricytochrome c is dependent on the concentration of the ferricytochrome c reducing systems of the red cells.

Discussion. The absorption spectrum of ferricytochrome c was changed towards that of ferrocytochrome c in the red cell suspensions (figure 1, A). This results demonstrates that extracellular ferricytochrome c was reduced by human red cells; the protein cannot permeate the red cell membrane. Accordingly, the following 2 possibilities may be suggested for the reduction mechanism; i.e. a) ferricytochrome c was indirectly reduced by an intracellular electron donor through the red cell membrane as Mishra and Passow[1] and Orringer and Roer[2] indicated in the case of ferricyanide reduction by human red cells; b) there are some electron transfer systems which are exposed at the outer surface of the red cell membrane.

The reduction of ferricytochrome c by red cells proceeds in the presence of deoxyglucose to the same extent as it does in the presence of glucose (figure 1, B); this results shows that the reduction of ferricytochrome c is not correlated to intracellular metabolism. The possibility that ascorbate-mediated transmembrane reducing systems are operating in the reduction of extracellular ferricytochrome c may be eliminated by the fact that the reduction was not affected in the presence of ascorbate oxidase, a scavenger of ascorbate. These results, therefore, support the 2nd view, that there are some ferricytochrome c reducing systems on the outer surface membrane.

Zamudio et al.[3] indicated that there is NADH oxidoreductase, which reduces both ferricytochrome c and ferricyanide, in the inner membrane of the red cells. Since NADH, NADPH, NAD, NADP, and FMN did not affect the rate of ferricytochrome c reduction (figure 1, A and B), the contri-

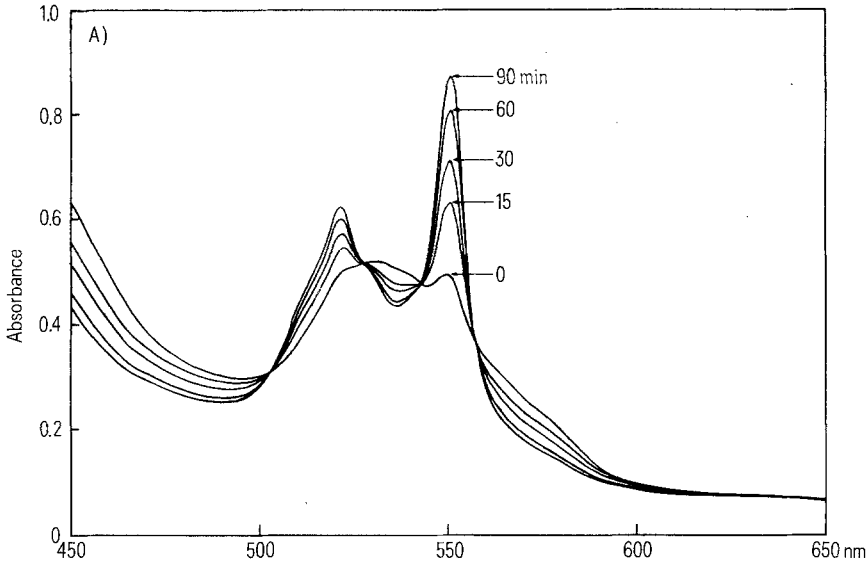
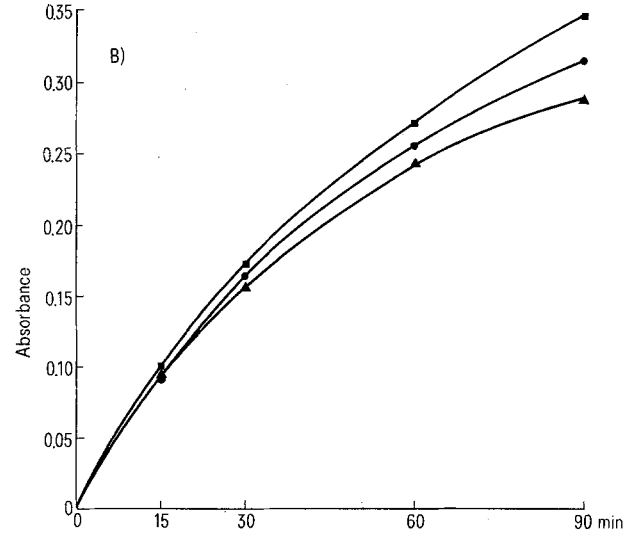


Fig. 1. Changes in absorption spectra between 450 and 650 nm and in absorbance at 550 nm during ferricytochrome c reduction by human red cells. *A* The absorption spectra in the control samples (no addition of reagents and enzymes) obtained at 0–90 min were measured between 450 and 650 nm. *B* Effects of deoxyglucose and ascorbate oxidase on the reduction of ferricytochrome c by human red cells. The optical density at 550 nm in the samples at each time was plotted against time. ●—●, control; ■—■, deoxyglucose (+) in the place of glucose; ▲—▲, ascorbate oxidase (+).



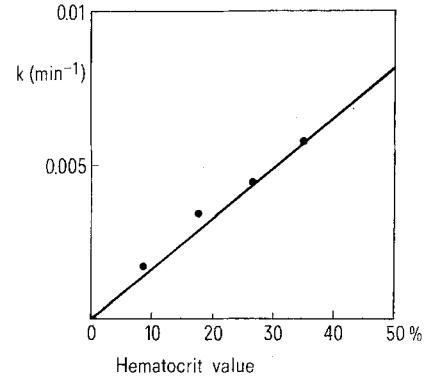


Fig. 2. Effects of varied concentrations of red cells on the reaction rate constants of ferricytochrome c reduction. The reduction of ferricytochrome c by human red cells (control) was analyzed by the increase in absorbance at 550 nm, varying the concentrations of red cells. From this result, the first-order reaction rate constants were obtained and plotted against hematocrit values.

bution of this enzyme to the ferricytochrome c reduction may be neglected.

It has been shown that covalent reagents such as NEM and PCMBS alter red cell membrane permeability through binding to membrane sulfhydryl groups[4]. It is not likely, however, that these sulfhydryl groups are involved in the reduction of ferricytochrome c, because the reduction rate was not altered in the presence of these reagents. Misra and Fridovich[5] showed that the superoxide anion, which is capable of reducing ferricytochrome c, is generated during autoxidation of hemoglobin. It seems that this anion does not participate in the reduction of extracellular ferricytochrome c, since the reduction mode was not altered in the presence of superoxide dismutase. Judging from the present results, it may be possible to say that the ferricytochrome c reducing systems are located on or exposed at the outer surface of the red cell membrane, though the characterization of the reducing systems must await further experiments.

1 R.K. Mishra and H. Passow, J. Membrane Biol. *1*, 214 (1969).
2 E.P. Orringer and M.E.S. Roer, J. clin. Invest. *63*, 53 (1979).
3 I. Zamudio, M. Cellino and M. Canessa-Fischer, Archs Biochem. Biophys. *129*, 336 (1969).
4 B. Shapiro, G. Kollman and D. Martin, J. Cell Physiol. *75*, 281 (1970).
5 H.P. Misra and J. Fridovich, J. biol. Chem. *247*, 6960 (1972).

Age correlated changes in midgut protease activity of the honeybee, *Apis mellifera* (Hymenoptera: Apidae)

D.E. Grogan and J.H. Hunt

Department of Biology, University of Missouri at St. Louis, St. Louis (Missouri 63121, USA), 29 February 1980

Summary. Forager (older) worker honeybees typically have lower midgut activity levels of chymotrypsin and trypsin than do house (younger) worker honeybees. A relation between the age correlated enzymic change and an age correlated decrease in pollen consumption is not clearly demonstrable.

We have previously reported that pollens collected by bees possess enzymic activities appropriate for protein digestion[1]. The well known age correlated changes in behaviour of the honeybee[2] offer an opportunity to further investigate the possible physiological roles of these pollen enzymes. Newly emerged and intermediate aged individuals largely confine their activities to the hive interior; older bees forage outside the hive. Pollen eating is pursued by younger and intermediate aged individuals[3]. DeGroot[4] demonstrated that both young and old worker bees can digest protein, but he also demonstrated that older bees are lower in total body nitrogen content and lower in weight than are younger bees. We report here quantitative differences in digestive enzyme activity in 2 age correlated samples of honeybee workers.

Materials and methods. 2 colonies of Italian strain honeybees were maintained in standard hives. Samples of house (younger) bees were taken from the upper interior of the hives; forager (older) bees were collected at the hive entrances. Midguts of 5–10 collected bees were removed

Table 1. Midgut weight and midgut chymotrypsin activities. Each sample is an average for midguts of 5–10 workers. H = house (younger) bees; F = forager (older) bees

Date (1979)		Midgut weight (mg)						Chymotrypsin (units/midgut)						Chymotrypsin (units/mg midgut protein)					
		Hive No. 1			Hive No. 2			Hive No. 1			Hive No. 2			Hive No. 1			Hive No. 2		
		H	F	F/H	H	F	F/H	H	F	F/H	H	F	F/H	H	F	F/H	H	F	F/H
June	5	10.5	9.3	0.89	13.2	10.5	0.80	1.87	1.70	0.91	3.70	1.50	0.41	2.57	2.40	0.93	4.47	2.48	0.55
	22	10.7	8.8	0.82	14.2	10.1	0.71	2.25	1.34	0.60	4.50	1.60	0.36	2.56	2.20	0.86	4.06	2.30	0.57
July	11	10.4	6.8	0.65	10.0	10.0	1.00	3.20	2.00	0.63	2.25	2.95	1.31	4.40	4.10	0.93	4.00	3.58	0.90
	26	11.4	8.7	0.76	14.2	9.30	0.65	4.60	3.00	0.65	5.30	3.70	0.70	4.70	4.30	0.91	4.56	4.90	1.07
August	9	12.7	9.3	0.73	11.5	10.0	0.87	5.45	2.75	0.50	4.05	3.10	0.58	4.80	4.72	0.98	3.46	3.70	1.07
	29	8.8	8.0	0.91	9.5	8.2	0.86	2.40	1.56	0.65	2.45	2.25	0.92	3.28	2.30	0.70	3.30	3.16	0.96
September	12	10.4	9.1	0.88	8.9	10.4	1.17	1.75	0.75	0.43	1.25	0.65	0.52	1.50	0.76	0.51	1.00	0.60	0.60
	28	9.2	7.8	0.85	10.5	8.6	0.82	2.50	1.70	0.68	1.70	1.90	1.12	3.30	2.50	0.76	2.54	3.00	1.18

Table 2. Honeybee midgut trypsin activities and chymotrypsin/trypsin ratios. Each sample is an average for midguts of 5 to 10 workers. H = house (younger) bees; F = forager (older) bees

Date (1979)		Trypsin (units/midgut)						Trypsin (units/mg midgut protein)						Chymotrypsin/trypsin			
		Hive No. 1			Hive No. 2			Hive No. 1			Hive No. 2			Hive No. 1		Hive No. 2	
		H	F	F/H	H	F	F/H	H	F	F/H	H	F	F/H	H	F	H	F
June	5	1.15	0.20	0.17	1.22	0.23	0.19	1.60	0.60	0.38	1.50	0.37	0.25	1.63	8.50	3.03	6.52
	25	0.60	0.24	0.40	1.40	0.25	0.18	0.75	0.40	0.53	1.30	0.40	0.31	3.75	5.58	3.21	6.40
July	11	0.95	0.22	0.23	1.05	0.60	0.57	1.33	0.45	0.34	1.90	0.85	0.45	3.37	9.09	2.14	4.92
	26	1.69	0.40	0.24	1.50	0.45	0.30	1.75	0.54	0.31	1.15	0.60	0.52	2.72	7.50	3.53	8.22
August	9	1.70	0.25	0.15	1.50	0.67	0.45	1.71	0.32	0.19	1.55	0.80	0.52	3.21	11.00	2.70	4.63
	29	0.65	0.70	1.08	0.85	0.51	0.60	0.85	1.05	1.24	1.15	0.75	0.65	3.69	2.23	2.88	4.41
September	12	0.53	0.16	0.30	0.75	0.26	0.35	0.50	0.32	0.64	0.65	0.53	0.82	3.30	4.69	1.67	2.50
	28	0.65	0.19	0.29	0.46	0.25	0.54	0.88	0.28	0.32	0.55	0.40	0.73	3.85	8.95	3.70	7.60