Table 1. CHARACTERISTICS OF G-6-PD FROM INDONESIA AND NEW GUINEA

Subject	Red cell G-6-PD activity units/g Hgb	K_m G-6-P* μM	2dG-6-P utilization† per cent
G-6-PD-deficient Indonesians			
T. S.	0·32	47 ± 1·3	5·5
T. L.	0·13	36 ± 3·6	4·3
A. B.	0·14	25 ± 4·8	9·4
L. H.	0·16	52 ± 2·7	4·0
Indonesian controls			
E. S.	5·77	49 ± 4·7	2·1
I. L.	5·12	53 ± 3·3	2·8
S. O.	5·31	55 ± 2·2	2·6
S. R.	6·35	48 ± 3·5	2·4
S. I.	4·36	58 ± 1·8	3·3
G-6-PD-deficient subjects from New Guinea ‡			
17 men	< 0·44	7·8 to 15·2 (range)	31 to 46 (range)

* ± Standard error.
† Expressed as a percentage of the rate at which the same amount of enzyme will utilize G-6-P. Average of duplicate determinations.
‡ Group II of an earlier study[4].

provided an even greater number of variants. People who are deficient in activity of red cell G-6-PD seldom have a complete deficiency of the enzyme. When subjected to purification and characterization, the enzyme from these people often exhibits decided abnormalities in several characteristics[2]. G-6-PD-deficient subjects from Canton have an enzyme that moves slightly faster than normal G-6-PD on electrophoresis in starch gel, and has Michaelis constants (K_m) that are significantly lower than normal. G-6-PD-deficient Negroes from North and South America and from western Africa have an electrophoretically fast enzyme with catalytic characteristics that are essentially normal. In contrast, the enzyme of most G-6-PD-deficient Caucasians from the Mediterranean area is electrophoretically normal but has decreased thermostability and alterations in many catalytic characteristics. A similar, if not identical, variant has been found in G-6-PD-deficient subjects from India[3] and New Guinea[4]. All seem to be sex (X)-linked.

These observations have prompted us to examine the characteristics of the enzyme from nine Indonesian subjects, consisting of: three G-6-PD-deficient men (T. S., T. L. and A. B.) and one G-6-PD-deficient woman of Indonesian–Chinese ancestry (L. H.), and five Indonesian subjects (controls) with normal activity for red cell G-6-PD (Table 1). The controls consisted of one woman (S. I.) and four men. Control E. S. is a brother of G-6-PD-deficient subject T. S.; Control I. L. is a brother of G-6-PD-deficient subject T. L. Methods for the purification and characterization at human red cell G-6-PD are described elsewhere[2,4]. K_m and standard errors of the K_m were determined from the enzymatic rate at six different concentrations of substrate by the statistical methods of Wilkinson[5].

The level of activity of G-6-PD in the haemolysates of all three G-6-PD-deficient Indonesian men was very low (Table 1). Their G-6-DP had normal migration during electrophoresis[4] in starch gel. In these respects, their enzyme was similar to G-6-PD Mediterranean. The utilization of 2-deoxyglucose-6-phosphate (2dG-6-P) and the K_m for G-6-P of their enzyme, however, were different from those found for seventeen G-6-PD-deficient men from New Guinea whose G-6-PD was of the "Mediterranean" phenotype (Table 1). Sufficient activity of G-6-PD was recovered from the red cells of three of the four G-6-PD-deficient Indonesian subjects to allow determination of pH optima curves. In contrast to the highly bimodal pH optimum curve obtained with the seventeen New Guinea subjects[4], the enzyme of only one of three G-6-PD-deficient Indonesian subjects exhibited activities that fell outside the normal range at various pHs (Fig. 1). Moreover, samples from T. S. and A. B. exhibited thermostabilities that were normal or slightly greater than normal when heated both alone and as mixtures[4] with normal enzyme. This observation is in contrast to the decreased thermostability of G-6-PD Mediterranean[6]. The difference was confirmed when thermostabilities were observed on T. S., a sample of G-6-PD Mediterranean and a mixture of the two. Characteristics of the G-6-PD from the woman of Indonesian–Chinese ancestry (L. H.) were similar to

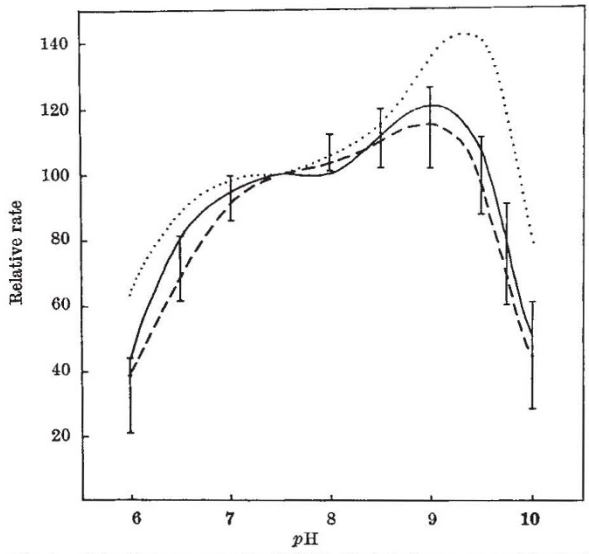

Fig. 1. pH optimum curves for G-6-PD. Rate is the average of duplicate determinations and is expressed as the percentage of the rate at pH 7·5. The vertical bars represent the mean ± 2 standard deviations for G-6-PD from twenty normal Caucasian men. ———, L. H.; . . . , A. B.; - - - -, T.S.

those of the three Indonesian G-6-PD-deficient men (Table 1), but her enzyme moved 5 to 7 per cent faster than normal G-6-PD during electrophoresis in starch gel. Part of the zone of activity extended into the normal zone. The properties of her G-6-PD are compatible with the possibility that she is a heterozygote for both the Canton and Indonesian variant of G-6-PD.

Fernandez and Fairbanks have recently reported a variant in the Philippines that is distinct from G-6-PD Mediterranean[7]. The Philippine and Indonesian variants appear to have similar levels of activity in red cells and similar thermostabilities. Whether the two variants are identical or not remains to be determined. These findings indicate that G-6-PD Mediterranean may not extend as a continuous chain from Sardinia to New Guinea. The chain is interrupted at least in Indonesia by the presence of another variant.

HENRY N. KIRKMAN

Department of Pediatrics,
University of North Carolina,
Chapel Hill, North Carolina.

LIE-INJO LUAN ENG

Institute for Medical Research,
Kuala Lumpur, Malaysia.

Received December 10, 1968.

1 Yoshida, A., *Proc. US Nat. Acad. Sci.*, **57**, 835 (1967).
2 Scientific Group, *WHO Tech. Rep. Ser.*, No. 366 (1967).
3 Azevedo, E., Kirkman, H. N., Morrow, A. C., and Motulsky, A. G., *Ann. Hum. Genet.*, **31**, 373 (1968).
4 Kirkman, H. N., Kidson, C., and Kennedy, M., *Hereditary Disorders of Erythrocyte Metabolism* (edit. by Beutler, E.), 126 (Grune and Stratton, New York, 1968).
5 Wilkinson, G. N., *Biochem. J.*, **80**, 324 (1961).
6 Kirkman, H. N., Schettini, F., and Pickard, B. M., *J. Lab. Clin. Med.*, **63**, 726 (1964).
7 Fernandez, M. N., and Fairbanks, V. F., *Mayo Clinic Proc.*, **43**, 645 (1968).

Antibody-like Activity to the 2,4-Dinitrophenyl Group in Normal Human Sera

VOLUNTEERS were injected with conjugates of 2,4-dinitrophenyl–human serum albumin (DNP–HSA)[1]. Sixty per cent of subjects developed rising titres of agglutinating activity to DNP coupled red cells after injection, but most normal pre-immunization sera also had some agglu-

tinating activity to DNP coupled cells. This activity was present in the fractions containing gamma globulin, was abolished by 2-mercaptoethanol, was specifically inhibited by the DNP haptenic determinant and was deficient in sera that were deficient in normal immunoglobulins—suggesting that most normal human sera contain antibody to the DNP group.

For haemagglutination assay, DNP coupled human group O red cells were prepared as described by Bullock and Kantor[2]. 0·1 ml. of a 1 per cent suspension of cells was added to 0·1 ml. of serial two-fold dilutions of sera in tubes. Although it was practical to read the settling patterns with high titred hyperimmune rabbit sera, this was less reliable with the low titred normal human sera. Instead of reading patterns therefore the cells were allowed to settle for 30 min and were then centrifuged at 1,000 r.p.m. for 2 min. The supernatant serum was then removed, 0·1 ml. of diluent was added and agglutination was read after gently tapping the tubes.

Of the fifty-six "normal" sera, twenty-eight came from male prisoner volunteers and twenty-eight were selected at random from various hospitalized patients. Of these fifty-six sera, 45 or 80 per cent had agglutinating activity toward DNP coupled cells. The titres varied from 1 : 4 to 1 : 64. Of thirteen human umbilical cord sera tested, eleven agglutinated DNP coupled cells, but the titres did not exceed 1 : 8 and the strength of agglutination was considerably less than that of the normal adult sera. Sera from patients expected to be deficient in normal immunoglobulins (hypogammaglobulinaemia, multiple myeloma) were also deficient in anti-DNP activity. Nine sera from patients with acquired or congenital hypogammaglobulinaemia were assayed. Only two of the nine had any agglutinating activity and the titre of these two did not exceed 1 : 8. The difference in the incidence of agglutinating activity between the hypogammaglobulinaemic and the normal groups was significant ($P = 0{\cdot}01$). One patient with type I dysgammaglobulinaemia (γG absent, with a high γM) had a titre of 1 : 64. Sera from twenty-nine patients with multiple myeloma were assayed. Only nine of the twenty-nine had agglutinating activity. The difference between these and the normal group was also significant ($P = 0{\cdot}01$). These data are illustrated in Fig. 1. None of four sera from patients with Waldenstrom's macroglobulinaemia had agglutinating activity.

Seven normal sera with activity were fractionated with ammonium sulphate. Precipitates were removed when 33 per cent and 50 per cent $(NH_4)_2SO_4$ saturated, and these and the supernates were analysed for gamma globulin content and agglutinating activity. The presence of γG, γA and γM globulins in the fractions was determined by radial diffusion in gel which contained specific antisera[3].

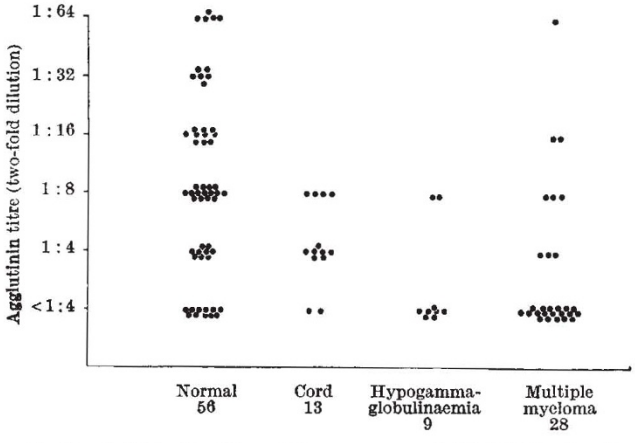

Fig. 1. Agglutination titres of human sera against 2,4-dinitrophenyl coupled group O human red cells.

85–95 per cent of the γG, 60–80 per cent of the γA and 60–75 per cent of the γM globulin were found in the 33 per cent fraction and the remainder was in the 50 per cent fraction. No gamma globulin was detected in the supernatants containing albumin and no agglutinating activity was present in these supernatants. All the agglutinating activity was recovered in the fractions containing gamma globulin and was distributed almost evenly between the 33 per cent and the 50 per cent precipitates. It was apparent that there was no correlation between the amount of γG and the agglutinating activity, that is, similar activity was present in some 33 per cent fractions which contained 95 per cent of the γG as in the 50 per cent fractions which contained the remaining 5 per cent. The differences in γA and γM content of the 33 per cent and the 50 per cent fractions were not as great as those of γG, and no definite correlation could be made between content of these and agglutinating activity.

Treatment with 0·2 M mercaptoethanol for 2 h abolished the agglutinating ability of normal sera, $(NH_4)_2SO_4$ fractions and the cord sera, suggesting that the activity is associated with the γM class of immunoglobulins.

To investigate specificity, the following DNP preparations were tested for their ability to inhibit agglutination of DNP coupled cells: DNP–HSA, DNP–lysine, DNP–haemocyanin (DNP–KLH), DNP–cellulose[4] and 2,4-dinitrophenol. Other preparations tested for inhibitory ability were the unrelated hapten altered proteins, azobenzenearsonate–HSA (R–HSA)[5], benzylpenicilloyl–HSA (BPO–HSA)[6] and the more closely related 2,4,6-trinitrophenyl–HSA (TNP–HSA).

Normal sera, ammonium sulphate fractions or cord sera were incubated at room temperature for 30 min with each compound before assay for agglutinating activity. A control of the same serum to which an equal amount of the corresponding protein, amino-acid or cellulose carrier had been added was assayed at the same time. The carriers alone had no inhibitory effect. The following molar concentrations were found to give complete or almost complete inhibition of agglutination: DNP–HSA $1{\cdot}2 \times 10^{-5}$ M, DNP lysine 2×10^{-4} M, 2,4-dinitrophenol $2{\cdot}7 \times 10^{-3}$ M. Addition of DNP–KLH in equivalent μg amounts of protein as DNP–HSA resulted in complete inhibition, although the precise molar concentration was not determined because of apparent breakdown of the haemocyanin molecule during conjugation. Adsorption of 0·5 ml. of sera with 20 mg of DNP cellulose removed all agglutinating activity. The unrelated hapten altered albumins, R–HSA and BPO–HSA and an R–cellulose adsorbent gave no inhibition. With the closely related TNP–HSA, results were variable. There was complete or almost complete inhibition with 7×10^{-5} M TNP–HSA in four of eleven sera. One of these was inhibited as well by $1{\cdot}2 \times 10^{-5}$ M TNP–HSA as by the same molar concentration of DNP–HSA. With the other seven sera there was no inhibition or only slight inhibition by 7×10^{-5} M TNP–HSA. Incubation of sera with DNP–HSA did not inhibit agglutination of unrelated systems, that is, ABO isohaemagglutination or agglutination of haemocyanin coated cells by immune human sera.

Eisen *et al.*, who demonstrated antibody activity to DNP in a γG myeloma protein, suggested that antibody activity be defined as specific binding by sites in the Fab domain of an immunoglobulin[7]. Although the anti-DNP activity demonstrated in normal sera by the work reported here occurs in the immunoglobulin containing fractions and is specific, the location of the binding site on the reactive molecule is not yet known. Positive identification of the active fraction as an immunoglobulin and the location of the binding site will require its isolation. The evidence presented here, however, strongly suggests that the activity to DNP and/or closely related groups is caused by antibody which may be regarded as "natural" antibody in that its presence cannot be ascribed to any obvious antigenic stimulus.

"Natural" antibodies have been directed against naturally occurring viral, bacterial, fungal, erythrocytic and other antigens[8] as distinct from an artificial low molecular weight determinant such as the DNP group described here. Anti-DNP activity may result from occult antigenic stimuli in the form of nitro-substituted benzene compounds which are used in dyes, herbicides, insecticides and in the manufacture of a variety of commonly used products. This possibility would dictate caution in the interpretation of results concerning immune responses assumed to be *de novo* to these and related compounds and perhaps to many potentially sensitizing substances. Another possibility is that this is a "preformed" antibody or recognition factor of the immune system which exists in the absence of any antigenic stimulus.

I thank the inmates of the Attica State Prison who participated in this study, and the warden and administration for their cooperation. This work was supported by a grant from the US Public Health Service.

MICHAEL W. BRANDRISS

Department of Medicine,
Rochester General Hospital,
Rochester, New York.

Received October 14, 1968.

[1] Brandriss, M. W., and Bullock, W. E., *Fed. Proc.*, **27**, 263 (1968).
[2] Bullock, W. E., and Kantor, F. S., *J. Immunol.*, **94**, 317 (1965).
[3] Fahey, J. L., and McKelvey, E. M., *J. Immunol.*, **94**, 84 (1965).
[4] Cheng, W. C., and Talmage, D. W., *J. Immunol.*, **97**, 778 (1966).
[5] Campbell, D. M., Garvey, J. S., Cremer, N. E., and Sussdorf, D. M., *Methods in Immunology*, 79 (Benjamin, W. A., Inc., New York, 1963).
[6] Brandriss, M. W., Smith, J. W., and Steinman, H. G., *J. Immunol.*, **94**, 696 (1965).
[7] Eisen, H. N., Little, J. R., Osterland, C. K., and Simms, E. S., *Cold Spring Harbor Symp. Quant. Biol.*, **32**, 75 (1967).
[8] Boyden, S. V., in *Advances in Immunology*, **5** (edit. by Dixon, jun., F. J., and Humphrey, J. H.) (Academic Press, New York, 1966).

Effect of Temperature on Resting Potential in Giant Axons of Squid

THE effect of temperature on the resting potential has been studied in a number of different preparations[1-3], and has attracted considerable theoretical interest. In the axons of the squid *Loligo forbesi*, the resting potential is essentially independent of temperature from 3° C to 20° C. Above this temperature, a decrease in resting potential has been observed, with a net fall of 10–15 mV at 35° C[1]. In the lobster axon[2], however, the results indicate that the resting potential is increased by 8–10 mV when the temperature is raised from 2° C to 16° C. According to more recent data obtained in lobster axons[3], the resting potential has a metabolic dependent component which, once eliminated by means of metabolic inhibitors, leaves the dependence of resting potential on temperature to be only the one expected if one assumes that the resting potential is a potassium ion electrode potential.

We describe here the effect of temperature on the resting potential in axons from the squid *Dosidicus gigas*, and relate this effect to a possible change in the structure of the axonal membrane.

Giant axons, with diameters ranging from 0·8–1 mm, were dissected and kept at 4° C until they were used. The technique for recording membrane resting and action potential by means of internal microelectrodes has been described[4,5]. The temperature of the external seawater bathing the axon in the chamber was allowed to increase or decrease gradually towards room temperature and was continuously monitored by a thermometer fixed in the chamber.

The recorded resting potentials were not corrected for junction potentials; the values obtained were within the range of −45 to −55 mV. Fig. 1 shows the results obtained with fifteen different axons. The vertical axis represents the differences in resting potential from the basal resting potential value taken at 17° C in the same axon.

The effect of temperature on the action potential was the same as that described in axons from other species; the duration of the action potential was greatly increased at low temperatures and the amplitude was slightly increased. The temperature was raised only to 24° C because at this point the axons tend to fail progressively in the conduction of the impulses.

In contrast to the results obtained with *Loligo* giant axons by Hodgkin and Katz[1], it was found that the resting potential decreased linearly as the temperature was increased from 3° C to 20° C. All changes in resting potential induced by changes in temperature were completely reversible provided that the temperature did not exceed 24° C. A depolarization, instead of a hyperpolarization, was obtained when the temperature of the media was raised. This decrease of resting potential with an increase in temperature contradicts the theoretically expected dependence, which states that the membrane potential should be proportional to the absolute temperature[6].

Also in contrast to the results obtained in lobster axons[3], the resting potential was not affected by metabolic inhibitors (0·2 mM 2,4-dinitrophenol or 10^{-4} M ouabain) even though these inhibitors, when added to the external seawater, strongly reduced the sodium efflux in axons from *Dosidicus*[7]. The effect of temperature on resting potentials remained essentially the same in the presence of these metabolic inhibitors, even after a long incubation period—30 min for 2,4-dinitrophenol and 45 min for ouabain.

Those axons which had been kept at 4° C for more than 24 h before the experiments were started gave results which deviate significantly from the straight line shown in Fig. 1; that is, the variations in resting potential with temperature were smaller (four axons). For this reason, only the results obtained with the axons that were used immediately after dissection were considered in Fig. 1.

The results reported here—that there is an increase in resting membrane potential as the temperature of the external seawater is decreased—suggest that changes occur in the axolemma which are independent of oxidative phosphorylation and sodium transport. We suggest that the axonal membrane structure becomes "more organized" at low temperatures and that this in turn determines an

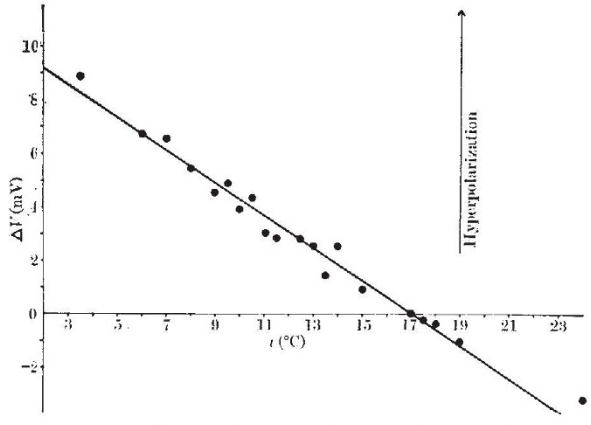

Fig. 1. Effect of temperature on resting potential. The temperature was varied by replacing the seawater bath around the axon. A continuous range of measurement was obtained by allowing the bath to warm or cool gradually towards room temperature. Each point represents at least four experiments. The ordinate represents the differences in resting potential from the basal resting potential value taken at 17° C in the same axon. Results from fifteen axons are included. The straight line obtained by least squares fitting of the experimental points is given by $\Delta V = -0{\cdot}65\,t + 10{\cdot}4$, where ΔV is the change in membrane resting potential and t is the temperature.