low standard physiochemical principles (*13*), it is not necessary to postulate special channels to interpret these results (*14*).

3) The concept of "specific blocking action of the sodium-carrier system" arose partly from the observed difference between the effect of TTX on action potentials produced in Ringer's (sodium-rich) solution and that in potassium-rich media (*4*). Although in the squid giant axon the effectiveness of TTX blockage differs according to the composition of the external media, the difference is only quantitative. Our experiments indicate that, at certain concentrations of TTX in the external medium, the action of TTX could be called "calcium-specific" (Fig. 2). However, this would obviously be inappropriate, since an increase in the concentration of TTX can also block the action potential in media containing sodium.

Our findings concerning the effects of TTX on squid giant axons do not support the separate channel hypothesis for excitable membranes.

There is considerable evidence indicating that exchange of cations at sites of the membrane macromolecules is the primary physicochemical event leading to the process of excitation (*14*). Strong binding of TTX to the charged sites on the external layer of membrane macromolecules would hinder the rapid exchange of small univalent and divalent cations, and would account for the observed effects of TTX. This explanation is consistent with the fact that TTX when applied intracellularly has no effect on the action potential (*15*).

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26 September 1966

Negro Variant of Glucose-6-Phosphate Dehydrogenase Deficiency (A^-) in Man

Abstract. *Glucose-6-phosphate dehydrogenase in erythrocytes of the Negro type associated with enzyme deficiency (A^-) was separated by chromatography on a carboxymethyl-Sephadex column from the electrophoretically indistinguishable Negro variant with normal enzyme activity (A^+). Quantitative immunologic neutralization tests indicated that the A^- enzyme had about the same enzymatic and serological activity as the A^+ and the normal (B^+) enzymes. The enzyme activity of the A^- variant in young erythrocytes was similar to that in young cells from normal individuals, although the activity of the A^- variant in unfractionated red cells was 10 to 15 percent of normal. These data indicate that the basic defect in the variant enzyme (A^-) is a structural mutation which causes more rapid degradation of the enzyme during erythrocyte aging.*

The erythrocytes of about 20 percent of Negro males have an electrophoretically rapid variant of glucose-6-phosphate dehydrogenase with normal enzyme activity (A^+). The cells of another 10 to 15 percent have a glucose-6-phosphate dehydrogenase which is electrophoretically indistinguishable from A^+ but has reduced (10 to 15 percent of normal) activity (A^-) (*1*). The levels of enzyme in nucleated tissues such as leukocytes and liver of A^- Negro males are normal or only slightly decreased (*2*). No consistent differences have been found between A^+ and A^- enzymes in enzymatic properties and immunological properties (*3*). Kirkman *et al.* suggested that enzyme deficiency in erythrocytes may be caused by a quantitative reduction in the number of the enzyme molecules (*4*). Luzzato and Allan observed differences in heat stability and elution patterns on diethylaminoethyl (DEAE)-Sephadex column chromatography between the A^+ and A^- enzymes (*5*).

Table 1. Enzyme activities in red cells of different age groups. The values are mean values and ranges (parentheses) of the number of units of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity per gram of hemoglobin in red cells obtained from three A^- and three normal (B^+) individuals.

Glucose-6-phosphate dehydrogenase		6-Phosphogluconate dehydrogenase	
Normal	A^- deficiency	Normal	A^- deficiency
Younger cells			
17 (16.1–18.2)	13 (12–15)	8.7 (7.0–10.2)	8.1 (6.5–10.5)
Total cells			
6.6 (4.4–7.8)	0.93 (0.76–1.1)	3.8 (3.3–4.2)	4.0 (2.7–5.1)
Older cells			
4.1 (3.4–4.9)	0.60 (0.4–0.7)	3.1 (2.8–3.4)	3.7 (2.5–4.6)

To find out whether the low activity in the red cells deficient in glucose-6-phosphate dehydrogenase from Negro males resulted from a structural abnormality producing a less-active enzyme molecule, from decreased rate of production, from an increased degradation rate, or from a combination of these effects, the following experiments were carried out.

The A^+ and A^- enzymes can be separated by DEAE-Sephadex column chromatography (*5*). The low recovery of the activity of A^- enzyme from the column could also be confirmed. Since the A^- enzyme was inactivated in the absence of nicotinamide-adenine dinucleotide phosphate (NADP), the low recovery of A^- enzyme from the DEAE-Sephadex column may have been partly due to loss of NADP by the anion exchanger during chromatography. Another chromatographic system—carboxymethyl cellulose–Sephadex column—gave a more effective separation of the A^+ and A^- enzymes (Fig. 1). The recovery of enzyme activity was better than 90 percent for each enzyme. These findings indicate structural differences between the A^+ and A^- enzymes.

Rabbit antiserum was prepared (*7*) by a series of subcutaneous injections

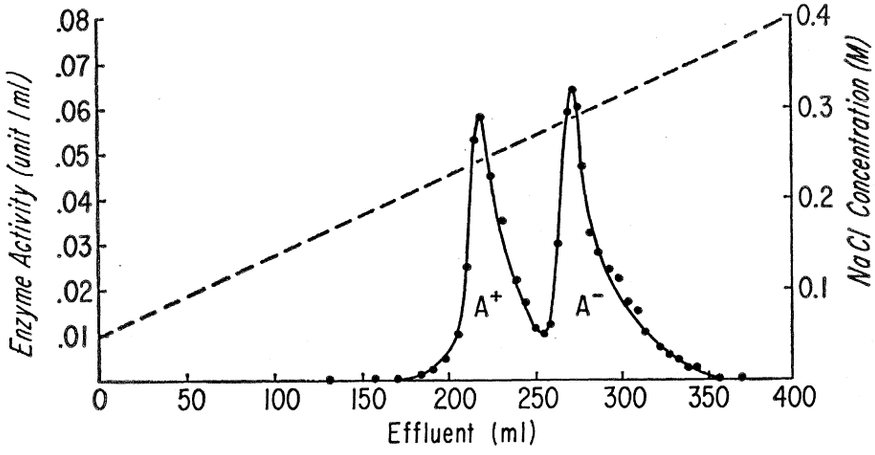

Fig. 1. Elution pattern of A^+ and A^- enzymes from a carboxymethyl-Sephadex column. A mixture of A^+ enzyme (1.65 units) and A^- enzyme (1.9 units) was placed on a carboxymethyl-Sephadex column (1 by 30 cm) buffered with 0.01*M* acetate buffer, *p*H 5.7, and eluted with linear gradient of NaCl concentration from 0.1 to 0.4*M*. Enzyme activity is expressed as the number of micromoles of NADP reduced per minute at 25°C.

with crystalline B^+ enzyme, which is homogeneous by various physicochemical criteria.

The purified A^+ and B^+ enzyme and partially purified A^+, B^+, and A^- showed no difference in neutralization behavior (Fig. 2). From the slope (Fig. 2) it was estimated that 1 ml of undiluted antiserum neutralized 8 to 9 units of each one of the different enzymes. Since the purified homogeneous preparations of B^+ and A^+ enzymes had a specific activity of 180 units per milligram of enzyme protein, 1 ml of antiserum absorbed about 50 μg of B^+ and A^+ enzyme protein (*8*). The B^+ enzyme is the homologous antigen for the antiserum used for this experiment; the antiserum, therefore, should have maximum capacity to absorb B^+ enzyme protein. Thus, the amount of A^- enzyme protein absorbed by 1 ml of the antiserum should not exceed 50 μg. This fact, together with the finding that 1 ml of the antiserum neutralized 8 to 9 units of A^- enzyme, indicated that the specific activity of A^- enzyme should be at least 180 units per milligram of enzyme proteins; that is, the A^- enzyme in each molecule must be as active as the normal (B^+) and A^+ enzymes.

If the serological activity of A^- enzyme were much lower than that of the normal (B^+) or A^+ enzyme (that is, lesser amounts of A^- enzyme protein were absorbed by the antiserum), the specific activity of A^- enzyme would have to be much higher than that of B^+ or A^+ enzyme, according to the equation shown in reference (*8*). This seemed highly unlikely. One may conclude that A^- enzyme has about the same serological activity and is enzymatically as active as the normal (B^+) and A^+ enzymes. Consequently, the low enzyme activity of deficient A^- subjects results from reduction in the number of enzymatically active A^- enzyme molecules in red cells.

No quantitative differences were found in the neutralization of the purified homogeneous enzyme preparations and the partially purified preparations which were prepared simply by eliminating hemoglobin from hemolyzates. This finding indicates that the partially purified preparation did not contain any other cross-reacting materials and that only the enzymatically active protein was serologically active (*9*).

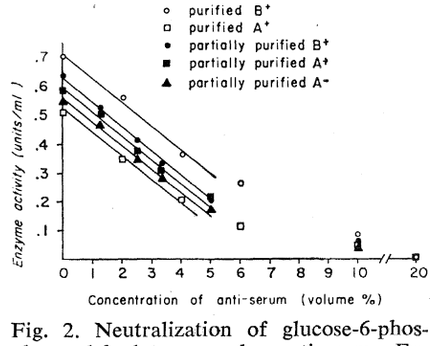

Fig. 2. Neutralization of glucose-6-phosphate dehydrogenase by antiserum. Enzyme samples were dissolved in 0.05*M* phosphate buffer, *p*H 6.8, containing $10^{-3}M$ EDTA, $10^{-3}M$ mercaptoethanol, and $2 \times 10^{-5}M$ nicotinamide-adenine dinucleotide phosphate and mixed with antiserum to B^+ of various dilution. Enzyme activity was assayed after the mixture had been kept at 30°C for 1 hour.

Erythrocytes were separated into "age" groups, according to their specific gravity, by the two-phase centrifugation developed by Danon *et al.* (*10*). Samples of fresh blood and phthalate ester mixtures of various densities were centrifuged (1.2 by 5 cm tubes) at 25,000 rev/min for 30 minutes at 25°C (Spinco model-L, L-50 rotor). Density of the phthalate ester mixture was selected so that in one tube about 5 percent of the cells representing the younger cells could be collected from above the fluid, while in the other, about 5 percent of the cells representing the older cells were collected from below the separating fluid. Fractionation and radioactivity measurement of erythrocytes obtained after labeling with Fe^{59} in vivo indicated good separation of the cells into different age groups (2670 count/min per milligram of hemoglobin in the top layer, 642 in the unfractionated cells, and 88 in the bottom layer, 7 days after labeling).

The normal younger cells (B^+) contained two to three times more (Table 1) glucose-6-phosphate dehydrogenase (as well as 6-phosphogluconate dehydrogenase) than the total cell population did, as has been reported by Marks *et al.* (*11*) and Brok *et al.* (*12*). In the red cells of Negro males (A^-), deficient in glucose-6-phosphate dehydrogenase, the younger cells contained more than ten times as much enzyme as the total cell population. Thus, the enzyme activity in the younger A^- cells was close to that of the normal (B^+) younger cells, even though the enzyme activity in unfractionated A^- red cells was only 10 to 15 percent in comparison with normals.

The mechanism of loss of enzyme activity with red cells aging is not clear. In vitro, the normal enzyme (B^+) was reversibly inactivated by dissociation into subunits by removal of bound NADP (*6*). The A^- enzyme was more easily (so far irreversibly) inactivated; that is, about 80 percent of enzyme activity was lost by dialysis for 3 days in the absence of NADP at 4°C. No serological cross reactions could be demonstrated in this inactivated enzyme by the quantitative neutralization test.

In summary, although the enzyme of the Negro-type deficiency (A^-) is structurally different from Negro variant with normal activity (A^+), the specific enzyme activity and the immunologic activity of the A^- enzyme molecule are very similar to those of

the A^+ and normal (B^+) enzymes. The enzyme deficiency in A^- red cells is principally caused by an increased rate of degradation rather than by a decreased rate of production of the enzyme.

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7. Antiserum to B^+ was obtained by injecting 0.4 mg of purified homogeneous B^+ glucose-6-phosphate dehydrogenase together with 0.5 ml of sodium alginate adjuvant and 0.5 ml of 0.1*M* $CaCl_2$. This dose was given weekly for 4 weeks.
8. The number of milligrams of enzyme protein absorbed by 1 ml of antiserum is equal to the number of units of enzyme activity neutralized by 1 ml of antiserum divided by the specific activity of enzyme.
9. No quantitative difference was found between the purified B^+, purified A^+, partially purified B^+, and partially purified A^+ by quantitative microcomplement fixation test (unpublished observation by Drs. V. Sarich and A. C. Wilson).
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Local Recognition of Histocompatibility Differences in Skin Grafts

Abstract. *Studies in rabbits of skin grafts tagged with tritiated thymidine indicate a greater proliferation of endothelial cells and fibroblasts at the site of an allograft than at that of an autograft as early as the first day after grafting. It appears, therefore, that allogeneic differences can be recognized and responded to locally almost at once. Labeled nuclear material is found to be transferred from the epithelial cells of skin grafts to host cells of the adjacent tissues. A mechanism therefore exists which might effect a local transfer of information on histocompatibility differences.*

It is generally accepted that first-set skin allografts are not distinguishable from autografts by morphological criteria for a number of days after grafting, the time depending on the degree of histocompatibility difference. Not until the immune response has progressed far enough to provide an observable difference in the degree of cellular infiltration and vascular development can the allograft be identified. In hundreds of skin cross-grafting experiments, using two outbred strains of rabbits, we have found this time to be hardly ever shorter than 4 days from the time of grafting, rejection occurring between 7 and 10 days. However, when graft sites were examined by microangiography, the impression was gained that the vascular reaction under and about a skin allograft is greater than that at an equivalent autograft in the same animal as early as 1 to 2 days after grafting.

In the present experiments an effort was made to substantiate this difference objectively by determining the uptake of labeled thymidine by the nuclei of endothelial cells and fibroblasts in the graft and graft bed. The demonstration of an almost immediate difference in the response to allograft and autograft would suggest that recognition of "nonself" may be very prompt and may take place locally. Such an early differential response has been reported in the goldfish with respect to scale transplants (*1*), and in the rabbit for hair follicles in transplanted skin (*2*).

Circular full thickness grafts of ear skin (1.7 cm in diameter) were exchanged between pairs of young rabbits (1 kg in weight) of two strains with presumed strong histocompatibility differences (New Zealand Whites and Chinchillas). Each rabbit received a similar autograft on its other ear at the same time. All surgical procedures were carried out aseptically. Grafts were left undressed. At 6 and 12 hours after grafting, and at daily intervals, rabbits were killed 2 hours after they had received an intravenous dose of tritiated thymidine (H3Th) (0.75 μc/kg of body weight, specific activity 3 c/mmole). Autoradiographs, exposed for 2 weeks, were prepared of tissue sections which included graft and graft bed. Cells with tritium-labeled nuclei, excluding cells of obvious epithelial origin, were counted from the epithelium to the cartilage, between grid lines (64 lines per inch) extending perpendicularly to the skin surface along the full length of the graft. Three to eight widely spaced tissue sections were counted in this way for each graft. The mean number of labeled cells per grid space was calculated for each section, and from those, the overall mean and variation for each graft. The allograft/autograft ratio of labeled cells was then determined for each animal.

As seen in Table 1, labeled nuclei were more numerous in the allograft than in the autograft in every animal. Apparently, then, the nonepithelial cells were proliferating at a greater rate in the allografts. Differences did not become statistically significant until 24 hours, but were in the same direction even at 6 and 12 hours. Results are presented for the first 4 days; thereafter standard morphological differences evoked by the immune response became obvious.

Although the time course of the reaction to the allograft might by itself suggest a secondary immune response, it is very unlikely that the recipient in each of the randomly selected animal pairs was presensitized to the donor tissue. Our findings are much more reasonably interpreted as indicating a local transfer of histocompatibility information from the outset of the healing of a tissue transplant. It is of interest that the reaction elicited is a mitogenic one.

"Antigenic recognition" in vitro was inferred by Möller (*3*) from the cytocidal effect of nonsensitized lymphocytes on allogeneic and semi-syngeneic target cells in the presence of phytohemagglutinin. However, in the absence of phytohemagglutinin, nonsensitized lymphocytes have a stimulatory effect on allogeneic target cells in vitro (*4*). We have observed in vivo a mitogenic effect upon the epithelium of skin allografts prior to graft destruction (*5*). The cells displaying labeling during the