locus (Figs. 5 to 19). Further explanation of the mode of action of 2*M* $MgSO_4$ or 2DG, or both, rests on a more definitive knowledge of the chemical composition of the cell walls of intact *H. capsulatum* yeast cells than is presently available.

Since these procedures are believed to be the first for the release of protoplasts at 37°C, they should be of particular value in further studies of yeasts pathogenic for humans. Previous investigators have never reported using osmotic stabilizing solutions above 1.3*M* (*3*), because this concentration appeared to be sufficient to maintain osmotically fragile protoplasts which had been induced by external enzymatic treatment. Whether molarities higher than 1.3 of $MgSO_4$ or other osmotic stabilizers will induce protoplast release in other actively budding yeasts remains to be determined.

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References and Notes

1. M. D. Berliner, *Sabouraudia* **6**, 111 (1968).
2. ——— and M. E. Reca, *Mycopathol. Mycol. Appl.* **37**, 81 (1969).
3. For a review of the studies on fungal protoplasts and discussion of terminology, see J. R. Villanueva, "Protoplasts of fungi," in *The Fungi*, G. C. Ainsworth and A. S. Sussman, Eds. (Academic Press, New York, 1966), vol. 2. Protoplasts can be formed either by the inhibition of cell wall synthesis or by the release of the intact cellular contents by various external enzymatic means. The latter is the only method reported for yeast protoplast formation and is the one discussed here.
4. Mycelial *H. capsulatum* stock strains G-184A, G-184B, G-186A, and G-186B (*1*) were freshly converted to the yeast phase on brain heart infusion agar plus cystine (BHI) at 37°C in an atmosphere of 5 percent CO_2 and subcultured on BHI for 48 hours. The growth from two slants of each strain was removed with trypticase soy broth (TSB) (*5*) and inoculated into flasks containing 50 ml of TSB. After being incubated for 48 hours at 37°C on a reciprocating shaker, 5 ml of the yeast suspension was transferred to fresh TSB and incubated for 18 hours more on the shaker. At 18 hours these cultures are at the beginning of the logarithmic growth phase (*5*), and portions (5 ml) were used as the test samples. The five $MgSO_4$ concentrations were made by adding the appropriate amounts of anhydrous $MgSO_4$ to TSB and then the solutions were sterilized by Seitz filtration. They were each inoculated with 5 ml of 18-hour yeast suspensions, incubated at 37°C on a reciprocating shaker, and observed daily for 5 days under medium phase contrast optics on a Zeiss photomicroscope. Photomicrographs were taken with Adox KB-14 film. The 2-deoxy-D-glucose (2DG) was from Mann Biochemicals.
5. M. E. Reca and C. C. Campbell, *Sabouraudia* **5**, 267 (1967).
6. B. F. Johnson, *Exp. Cell Res.* **50**, 692 (1968).
7. M. D. Berliner, *Sabouraudia* **5**, 26 (1966).
8. M. R. Edwards, E. L. Hazen, G. A. Edwards, *J. Gen. Microbiol.* **20**, 496 (1959).
9. Supported by NIAID research grant No. 5 RO1 AI07520-04 and NIH Fund for Research and Teaching in Tropical Public Health No. 5799-2.

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Penicillin as Epileptogenic Agent: Its Effect on an Isolated Neuron

Abstract. *Penicillin induces partial depolarization and increased excitability of the neuronal membrane of crayfish stretch receptor. Such effects suggest that the epileptic focus created by the topical application of penicillin to the mammalian cerebral cortex may result from the lowering of the threshold for impluse initiation by excitatory synaptic action within the neuron population.*

At high concentrations penicillin consistently produces seizures when topically applied to the cerebral cortex and, therefore, has become one of the most widely used epileptogenic agents. The electrocortical activity of the epileptic focus is characterized by a repetitively recurring, isolated, high-voltage negative wave—the interictal "spike" of the electroencephalograph. Simultaneously with the interictal "spike," the transmembrane potential of the neurons within the focus undergoes a typical depolarization of several millivolts which lasts as long as 100 msec. Although these events are quite stereotyped, the mode of action of penicillin as an epileptogenic agent is not clearly understood. The paroxysmal activity in the neurons of an epileptic focus might be produced by a variety of mechanisms. For example, there may be changes in the utilization of the preexisting neuronal circuitry within the population making up the focus, or some radical changes in the mode of impulse initiation in individual neurons might account for the epileptic activity (*1*).

To study the effect of penicillin on the electrical activity of an isolated neuron which is free of the complications introduced by neuronal circuitry, we chose the crayfish muscle stretch receptor. This preparation has been extensively studied physiologically (*2*). Also, the effect of another convulsant agent, strychnine, on this preparation is known (*3*) so that comparison can be made.

The slow-adapting stretch receptor was isolated and held unstretched in a plexiglass chamber containing Van Harreveld's crayfish saline (*4*). The axon, covered with Vaseline to prevent dehydration, was suspended by platinum electrodes for external recording and antidromic stimulation. Glass microelectrodes filled with 3*M* KCl were used to penetrate the soma. In some experiments, microelectrodes filled with potassium citrate were also used. No differences were noted. A bridge circuit permitted simultaneous recording and stimulation. An Ag–AgCl electrode connected to the bath through a KCl agar bridge served as ground. This reduced the large junction potential which developed when the concentrated penicillin solution was introduced into the chamber. Two procedures were used to measure the membrane potential. In those experiments where the penicillin solution was not rinsed away, the potential was calculated with the reading obtained after the withdrawal of the microelectrode from the neuron as zero potential. Where the penicillin solution was replaced with saline, the microelectrode was withdrawn, penicillin was again introduced into the chamber, and the junction potential was measured. The measurements usually coincided when both methods were used in the same preparation. The values reported are corrected for the junction potential. The sodium concentration in the crayfish saline was appropriately reduced to allow for the sodium in the penicillin salt. Both the crayfish saline and the penicillin solutions were oxygenated. To change the solution in the perfusion chamber, a volume of fluid at least 50 times that of the chamber volume (1.6 ml) was allowed to flow.

The effect of penicillin was related to the concentration. Perfusing with a solution containing 20,000 international units (I.U.) per milliliter (0.034*M*) produced (i) a decrease of the membrane potential difference, (ii) an increase of membrane excitability, and (iii) a decrease of activation of the soma by the antidromic spike.

An increase of a few millivolts in the transmembrane potential was observed in some preparations immediately after the start of perfusion (Fig. 1, B and C). This hyperpolarization lasted a few minutes and then, in all cases, disappeared. There followed a slow depolarization which, if the preparation was not rinsed with crayfish saline, brought the membrane to zero potential after 90 to 180 minutes.

The excitability of the membrane, as tested with pulses of constant intensity of outward current through the microelectrode, decreased during the early part of the hyperpolarization (Fig. 1B),

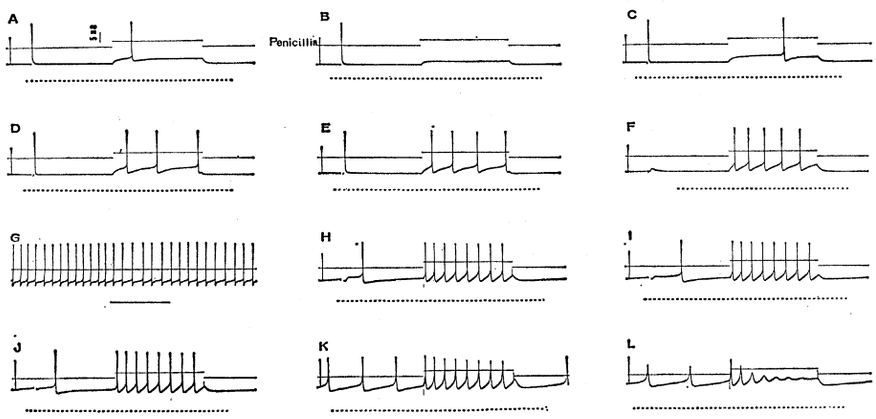
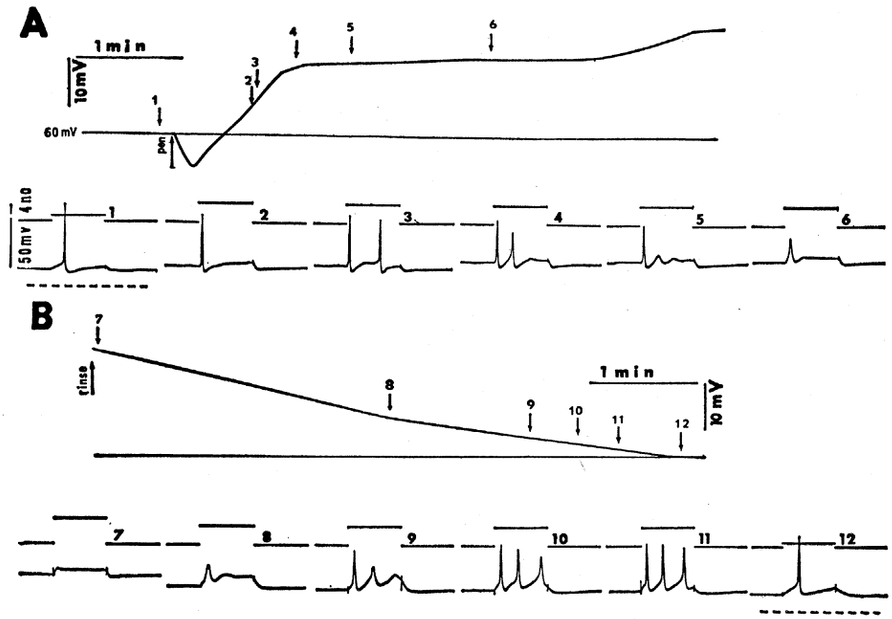


Fig. 1 (top left). Perfusion with sodium penicillin G (20,000 I.U./ml) solution. The vertical bars at the beginning of the sweep represent a 50-mv calibration. The bottom horizontal short bars in each sweep represent 10 msec each, except that in (G), which represents 1 second. The amount of outward current injected through the microelectrode was kept constant. The spike before the direct stimulation was obtained by antidromic stimulation. In (F) only an axonal spike was present. In (H), (I), and (J) the intensity and duration of the antidromic stimulation was increased; a depolarization followed the axonal spike and an antidromic spike was obtained with different delays. (A) Control, membrane potential 65 mv. The perfusion was started between (A) and (B). The respective membrane potentials and time intervals from the start of perfusion for each example were: (B) 68 mv, 2 minutes; (C) 68 mv, 3 minutes; (D) 64 mv, 15 minutes; (E) 62 mv, 18 minutes; (F) 61 mv, 25 minutes; (G) 58 mv, 42 minutes; (H), (I), and (J) 52 mv, 50 minutes; (K) 66 minutes; and (L) 68 minutes.



but returned to control during the last part of hyperpolarization (Fig. 1C) and increased with depolarization (Fig. 1, D to H). In some preparations, this increase of excitability was observed even during the hyperpolarization, before the membrane potential had returned to control value (preperfusion level). When membrane resistance was measured by intracellular injection of pulses of constant inward and outward current, either no change or an increase was observed during the late phase of the hyperpolarization and the early part of the depolarization. When the membrane potential approached the triggering potential, it became unstable and spontaneous firing occurred (Fig. 1G). The firing was maintained until the depolarization was great enough to arrest spike electrogenesis. Spike arrest oc-

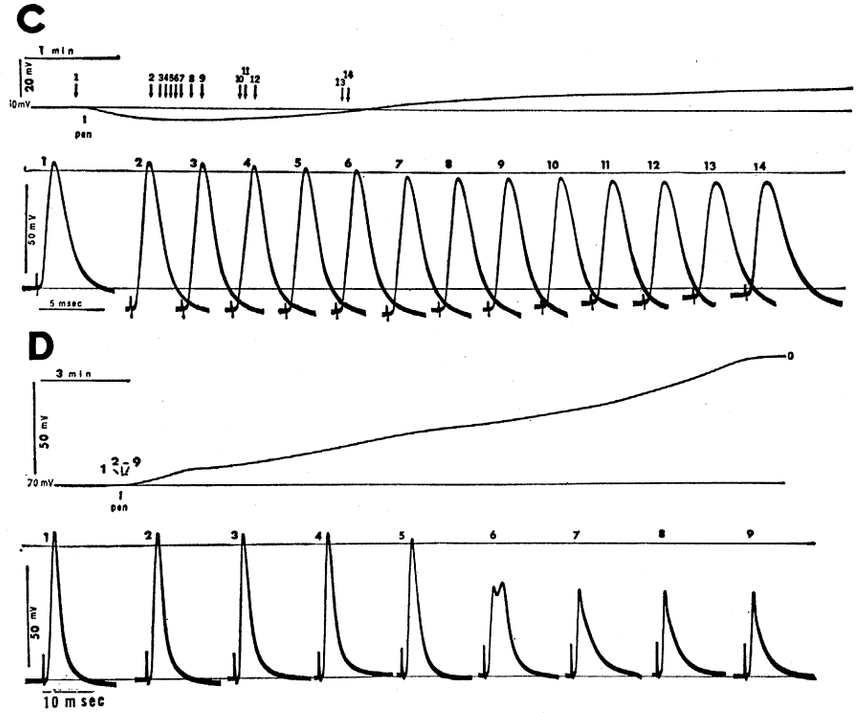


Fig. 2 (bottom left). Perfusion with sodium penicillin G (100,000 I.U./ml) solution. The changes in membrane potential are plotted in (A), (B), (C), and (D). Each insert is numbered to correspond to the numbered arrow. (A) and (B) are from the same receptor and the plottings are continuous in time. In this receptor only direct stimulation was used. The stimulating outward current was increased during the initial hyperpolarization to obtain the spike and was kept constant until insert 7, when it was increased. Then it was decreased to the same intensity as inserts 2 to 6. The current of insert 12 is the same as that of insert 1, the control before the penicillin perfusion. (C) and (D) illustrate the changes occurring in the antidromic spikes of two different receptors. No action potentials were obtained after spike 14 in (C) and spike 9 in (D).

curred at a membrane depolarization which, in previous tests with outward currents through the microelectrode, was still capable of producing spikes.

The threshold for antidromic activation was also affected, and it was necessary to increase the duration or the intensity, or both, of the stimulating current to maintain the antidromic response. At control intensities, only the axonal spike appeared (Fig. 1F) which was often followed by a long depolarization of a few millivolts and a spike occurring after a variable delay (Fig. 1, H to J).

If the penicillin solution was replaced with saline within 30 to 40 minutes, that is, during the period of increased excitability of the receptor but before the spontaneous firing had occurred, the membrane immediately depolarized further and spontaneous firing began. The membrane depolarization remained at this new value for hours. However, the conditions for antidromic activation were reestablished at the control levels following penicillin removal.

The perfusion with higher concentrations of penicillin accelerates the rate of development of the events described. The initial hyperpolarization lasted 1 minute at most, and the rate of depolarization was faster (Fig. 2A). The arrest of antidromic activation occurred in 1 or 2 minutes. This is shown in Fig. 2, C and D, where no action potentials were obtained after the last ones illustrated. The somatic spike triggered by direct stimulation was arrested before any sign of increased excitability could be observed. It was possible to trigger a spike only by applying large amounts of outward current. The electrogenesis of somatic spikes was completely arrested at a depolarization of only a few millivolts (Fig. 2A). No spontaneous firing was observed with a solution of 100,000 I.U./ml (0.175*M*) and only random spikes occurred with 50,000 I.U./ml of solution. However, if these solutions of high concentrations were removed within a few minutes (even after the characteristic changes had already taken place), the membrane potential, the excitability, and the antidromic activation soon returned to control values (Fig. 2B).

The behavior of the antidromic spike during perfusion with high concentrations of penicillin presents some interesting features. If an initial hyperpolarization occurs, the height of the spike becomes larger but the overshoot remains unchanged (spike No. 2, Fig. 2C). This may be an indication that there is no reduction of membrane resistance (at least in those portions of the neuronal membrane where the somatic spike is generated). However, when the membrane began to depolarize, the overshoot progressively decreased and the base of the spike became wider (spikes 4 to 14, Fig. 2C), until finally the spikes were arrested. Note that the arrest of the spike occurred even before the membrane potential regained control value. If there was no initial hyperpolarization, the spike size and overshoot were initially unchanged (spikes 2 to 4, Fig. 2D), then a reduction of the overshoot occurred (spike 5, Fig. 2D). There was then a delay of somatic activation (spike 6, Fig. 2D) as shown by the clear breaking of the spike into its two components, IS-SD (initial segment, soma dendrite). Eventually, only the IS spike was triggered (spikes 7 to 9, Fig. 2D), until it, too, was arrested so that only the propagation of the axonal spike could be observed (not shown in the figure).

Thus the direct effects of penicillin on the membrane of an isolated neuron are, at the concentrations used, an initial hyperpolarization, a later sustained depolarization associated with increased excitability and final arrest of spike electrogenesis. Such effects are much easier to see and simpler to interpret than those seen in cortical cells where circuitry evidently is responsible for the explosive depolarizing responses.

These effects of penicillin on the crayfish stretch receptor are quite different from those reported for strychnine (*3*), which induced sustained abrupt depolarizations and grouped firing.

Some of our observations, such as the antidromic spike arrest, the time course of the slow depolarization, and the spontaneous firing, are similar to those reported by Obara and Grundfest (*5*) who treated the same preparation with lithium solution. These authors suggested the possibility of sodium pump inactivation to account for the effects. Most of our observations with penicillin are likewise explainable if the sodium pump of the crayfish stretch receptor is assumed to be impaired in its ability to maintain a full resting membrane potential and normal ionic gradients. We have performed preliminary experiments with ouabain, an agent believed to selectively block the sodium pump, and observed an apparent similarity between ouabain and penicillin effect.

Although spike electrogenesis involves an interplay among several processes, the block of the spike production in the soma in these experiments cannot be attributed to the depolarization since it can occur even before the depolarization takes place (Fig. 2C). Arrest of sodium extrusion would decrease the diffusional electromotive force across the membrane because of accumulation of intracellular sodium. One would observe first a decrease of the overshoot (Fig. 2, C and D) and finally a complete arrest of the spike. The loss of normal ionic gradient could also explain the slow depolarization and the increased excitability. However, at this time we do not have explanations for the increased depolarization when the penicillin solution (20,000 I.U./ml) was replaced with saline in 20 to 30 minutes, and for the initial hyperpolarization observed with all the concentrations tested.

The increase of excitability of the neuronal membrane of the crayfish stretch receptor suggests a possible mechanism for the apparently different effect of the drug when it is applied topically to the mammalian cerebral cortex. If the assembly of neurons exposed to the drug develops the same depolarization and increased excitability observed in the stretch receptor, the threshold for impulse initiation by excitatory synaptic action would be lowered. This effect would greatly potentiate recurrent excitatory pathways of the cortex so that recruitment of more neurons could take place until a paroxysmal discharge occurs. The failure to identify definite changes in resting membrane potential in cortical neurons during the interictal spike-free period may be due to technical difficulties. In fact, because of the inability to compare the true resting membrane potential before and after the application of the epileptogenic agent, the limited degree of membrane depolarization that is sufficient to enhance the gain of the recurrent excitatory system may have escaped identification.

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References and Notes

1. H. Matsumoto and C. Ajmone Marsan, *Exp. Neurol.* **9**, 286 (1964); D. A. Prince, *ibid.* **21**, 464 (1969); *Electroencephalogr. Clin. Neurophysiol.* **26**, 476 (1969); H. Matsumoto, G. F. Ayala, R. J. Gumnit, *J. Neurophysiol.* **32**, 688 (1969); M. A. Dichter and W. A. Spencer, *ibid.*, p. 649.
2. C. Eyzaguirre and S. W. Kuffler, *J. Gen. Physiol.* **39**, 87 (1955); C. A. Terzuolo and Y. Washizu, *J. Neurophysiol.* **25**, 56 (1961); C. Edwards, C. A. Terzuolo, Y. Washizu, *ibid.* **26**, 948 (1963); S. Nakajima and K. Takahashi, *J. Physiol.* **187**, 105 (1966).
3. Y. Washizu, G. W. Bonewell, C. A. Terzuolo, *Science* **133**, 333 (1961).
4. A. Van Harreveld, *Proc. Soc. Exp. Biol. Med.* **34**, 428 (1936).
5. S. Obara and H. Grundfest, *J. Gen. Physiol.* **51**, 635 (1968).

6. Supported by NINDB grants NB 5466 and NB 7192 and the Epilepsy Foundation of America. We thank the Upjohn Co. for supplying us a uniform batch of sodium penicillin G. We also gratefully acknowledge the suggestions of Drs. C. Edwards and W. A. Spencer during the preparation of the manuscript.

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Ranks of Donor-Recipient Histocompatibility for Human Transplantation

Abstract. *Correlation of leukocyte groups with skin and renal allograft survival indicates that ranks of histocompatibility based upon current genetic concepts of the HL-A system may provide an approach to the selection of optimally compatible subjects for clinical organ transplantation. Such ranks may be expressed as a net histocompatibility ratio (NHR) between prospective donors and recipients. The best clinical results have been when this ratio is of 0.5 to 1. Donor-recipient compatibility situations where the ratio was 0.25 or less have been associated with a high incidence of transplant failure, regardless of whether the organ source was a living, related donor or a cadaver donor.*

As a result of genetic studies of the HL-A system of histocompatibility two subloci have been isolated in the HL-A region of the human chromosome; the antigens HL-A1, HL-A2, HL-A3, HL-A9, Da 15, and Da 17 being determined at the first sublocus, and antigens Da 4, HL-A5, HL-A7, and HL-A8 at the second sublocus (*1*). Four additional HL-A antigens [Da 6, Da 9 (*2*), Da 18, HN] may be included on the second sublocus (*3*). The antigens determined at each of the subloci occur as alternative alleles. It is also probable that a large majority of the HL-A antigens capable of conditioning the survival of human organ transplants is included in these two subloci (*4*).

Calculations based on donor-recipient compatibility for the ABO erythrocyte group antigens and the HL-A antigens listed above indicate that a minimum of 500 prospective recipients may be required in order to give a cadaver organ donor a 95 percent chance of being transplanted to a serologically compatible host. Terasaki has indicated that such a waiting list might actually be as high as 1000 subjects (*5*). As progress is made in the isolation of additional HL-A alleles, the size of such a waiting list, if based upon this type of calculation, will inevitably grow in direct proportion to increases in the number of known alleles. Concurrent advances in our understanding of the genetic determinants of the HL-A system may, however, provide for an alternative approach to the selection of donors. Such an approach is based upon the notion that donor-recipient antigenic identities at the two known HL-A subloci are of the greatest importance in conditioning allograft survival. The results of experimental (*6*) and clinical transplantation studies (*7*) support this concept.

During these studies an attempt was made to correlate the survival time of skin allografts from sibling to father with the HL-A genotypes of donor and recipient. The actual ratio of antigenic identities (ID) to incompatibilities (IN) at each of the known HL-A subloci may provide a particularly reliable statistical index of compatibility between donor and recipient (*3*). In an extension of this series to 135 skin allografts from sibling to father, 33 transplants survived for 13.5 days or more (group A), and 27 grafts were rejected on or before day 10 after operation day (group B). In 30 of the 33 grafts (90 percent) in group A, the number of antigenic identities between donor and recipient was as great or greater than the number of incompatibilities at each of the two known HL-A subloci. In contrast, 17 of 27 (63 percent) of the grafts in group B were applied under circumstances where the number of incompatibilities exceeded the number of identities. Genotyping of the subjects in group A has indicated subsequently that 42 percent of the genes at the HL-A subloci in these subjects governed antigens which constituted incompatibilities between donor and recipient, and 36 percent of such genes provided for donor-recipient identities. In contrast, similar genotype studies in group B indicated that 77 percent of the genes at the two HL-A subloci determined antigenic incompatibilities, and only 3 percent of the genes provided for donor-recipient identities. The number of donor-recipient identities was therefore 12 times greater in group A (that is, the grafts accorded the longest survival times) than in group B (the grafts which underwent early rejection). These studies have also yielded evidence that the currently known HL-A antigens may be roughly equipotential in their immunogenicity (*4*).

Review of the observed ratio of identities to incompatibilities (ID/IN) in donors and recipients of 76 renal transplants obtained from donors that were living and related and from cadaver donors (*8*) has yielded results that paral-

Table 1. Calculation of net histocompatibility ratios (NHR) for human transplantation. Donor and recipient are compared at two known HL-A subloci.

Situation 1	Situation 2
All antigens determined by four genes at the two subloci have been detected	Not all of the antigens determined at each of the four genes at the two subloci have been determined
a) Four antigens have been detected in each subject	a) Unknown antigens not yet detected at that sublocus
or	*or*
b) Less than four antigens have been recognized but the subjects are homozygous at the sublocus in question on the basis of family studies	b) Less than four antigens have been detected in a subject where homozygosity at any one given sublocus cannot be confirmed by family studies
There are clear-cut identities (ID) and incompatibilities (IN) at each sublocus	There may be some clear-cut identities (ID) and incompatibilities (IN) at each sublocus
	But also
	Each PR situation may be either an ID or an IN between donor and recipient
NHR = ¼ × (total ID/total IN)	NHR = ¼ × (avg. of total ID/total IN) taking each PR into consideration as either an ID or an IN