approaches based on exploitation of such a biochemical difference—for example, by selective inhibition of asparagine utilization and biosynthesis in tumors.

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References and Notes

1. J. G. Kidd, *J. Exp. Med.* **98**, 565, 583 (1953).
2. P. A. Herbut and W. H. Kraemer, *Amer. J. Pathol.* **34**, 767 (1958); K. S. Kwak, E. Jameson, R. M. Ryan, H. M. Kurtz, *Cancer Res.* **21**, 44 (1961); E. Jameson, H. Ainis, R. M. Ryan, *Science* **124**, 980 (1956).
3. E. A. Boyse, L. J. Old, E. Stockert, *Nature* **198**, 800 (1963); E. A. Boyse, L. J. Old, H. A. Campbell, L. T. Mashburn, *J. Exp. Med.* **125**, 17 (1967).
4. J. D. Broome, *Nature* **191**, 1114 (1961); *J. Exp. Med.* **118**, 99, 121 (1963).
5. L. J. Old, E. A. Boyse, H. A. Campbell, G. M. Daria, *Nature* **198**, 801 (1963); N. D. Holmquist, *Proc. Soc. Exp. Biol. Med.* **113**, 444 (1963); L. T. Mashburn and J. C. Wriston, Jr., *Biochem. Biophys. Res. Commun.* **12**, 50 (1963); H. A. Campbell, L. J. Old, E. A. Boyse, *Proc. Amer. Assoc. Cancer Res.* **5**, 10 (1964); H. M. Suld and P. A. Herbut, *J. Biol. Chem.* **240**, 2234 (1965); T. O. Yellin and J. C. Writson, Jr., *Science* **151**, 998 (1966); R. C. Hiramoto, C. Tate, M. Hamlin, *Proc. Soc. Exp. Biol. Med.* **121**, 597 (1966).
6. L. T. Mashburn and J. C. Wriston, Jr., *Arch. Biochem. Biophys.* **105**, 450 (1964); H. A. Campbell, L. T. Mashburn, E. A. Boyse, L. J. Old, *Biochemistry* **6**, 721 (1967); W. C. Dolowy, J. Cornet, D. Hensen, R. Ammeraal, *Proc. Soc. Exp. Biol. Med.* **123**, 133 (1966); J. Roberts, M. D. Prager, N. Bachynsky, *Cancer Res.* **26**, 2213 (1966); J. H. Schwartz, J. Y. Reeves, J. D. Broome, *Proc. Nat. Acad. Sci. U.S.* **56**, 1516 (1966).
7. L. J. Old, E. A. Boyse, H. A. Campbell, R. S. Brodey, J. Fidler, J. D. Teller, *Cancer* **20**, 1066 (1967).
8. J. M. Hill, J. Roberts, E. Loeb, A. Khan, A. MacLellan, R. W. Hill, *J. Amer. Med. Assoc.* **202**, 882 (1967).
9. H. F. Oettgen, L. J. Old, E. A. Boyse, H. A. Campbell, F. S. Philips, B. D. Clarkson, L. Tallal, R. D. Leeper, M. K. Schwartz, J. H. Kim, *Blood* **30**, 852 (1967); *Cancer Res.* **27**, 2619 (1967).
10. A. Meister, Ed., *Biochemistry of the Amino Acids* (Academic Press, New York, ed. 2, 1965), vol. 1, pp. 457–60.
11. J. M. Ravel, S. J. Norton, J. S. Humphreys, W. Shive, *J. Biol. Chem.* **237**, 2845 (1962).
12. L. Levintow, *Science* **126**, 611 (1957).
13. M. K. Patterson, J. Orr, G. Orr, *Biochem. Biophys. Res. Commun.* **26**, 228 (1967).
14. B. K. Madras and A. Meister, unpublished, 1967.
15. A. Meister, in *The Enzymes* (1962), vol. 6, p. 247.
16. J. D. Broome and J. H. Schwartz, *Biochem. Biophys. Acta* **138**, 637 (1967).
17. A. Meister, Ed., *Biochemistry of the Amino Acids* (Academic Press, New York, ed. 2, 1965), vol. 2, pp. 607–17.
18. L. J. Old, E. A. Boyse, E. Stockert, *J. Nat. Cancer Inst.* **31**, 977 (1963); *Cancer Res.* **25**, 813 (1965). Sensitive and resistant lines of 6C3HED and P1798 were kindly supplied by L. Mashburn, Hospital for Joint Diseases and Medical Center, New York; L5178Y, by J. H. Burchenal, Sloan-Kettering Institute for Cancer Research, New York.
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Virus-Induced Hemolysis in Erythrocytes Deficient in Glucose-6-phosphate Dehydrogenase

Abstract. *Red cells from individuals deficient in glucose-6-phosphate dehydrogenase undergo increased autohemolysis when incubated in the presence of influenza-A virus. Normal red cells, but not those from individuals deficient in glucose-6-phosphate dehydrogenase, show increased activity of the hexose monophosphate shunt in the presence of the virus. This increase in shunt activity appears to be related to oxidation of cellular sulfhydryl groups.*

Increased hemolysis, as evidenced by jaundice and significant anemia, has been observed in individuals deficient in glucose-6-phosphate dehydrogenase (G-6-PD) and showing clinical signs of viremia and certain bacterial infections in the absence of any possibly offending drug (*1*). The observation that a patient severely deficient in G-6-PD had a recurrent fall in hemoglobin level of at least 2 g during upper respiratory infections prompted this investigation.

The initial experiments were designed to determine whether the hemolytic process associated with viremia could be reproduced in vitro. Red cells from three patients with severe G-6-PD deficiency (less than 10 international units per gram of hemoglobin) were washed three times in isotonic saline and then incubated at 37°C for 48 hours in the presence of serial titers of an influenza virus (*2*). Hemoglobin was measured in the supernatant by a benzidine method (*3*). The values obtained were calculated as percentage hemolysis, as in the autohemolysis test (*4*). In the absence of virus, normal red cells undergo less than 2 percent autohemolysis in 48 hours.

From the results of a typical experiment (Fig. 1) it is evident that concentrations of virus higher than 10^3 particles per cubic millimeter are associated with increased autohemolysis in cells deficient in G-6-PD. The addition of glucose had but little effect on the hemolytic action of the virus. No complement was present, and viral-induced agglutination of the red cells was not observed. Increased numbers of Heinz bodies, however, were observed in cells deficient in G-6-PD incubated with virus. Prior inactivation of the virus by either heat or specific antibody abolished completely the hemolytic activity.

Because this clinical syndrome of hemolysis was associated with a biochemical defect in the hexose monophosphate shunt, the next experiment involved an investigation of this pathway. A relatively simple technique (*5*), for evaluation of this pathway in the intact red cell, uses as an index of hexose monophosphate shunt metabolism the production of $C^{14}O_2$ from glucose labeled in the 1-carbon position. Since in the mature erythrocyte the shunt represents the only known locus

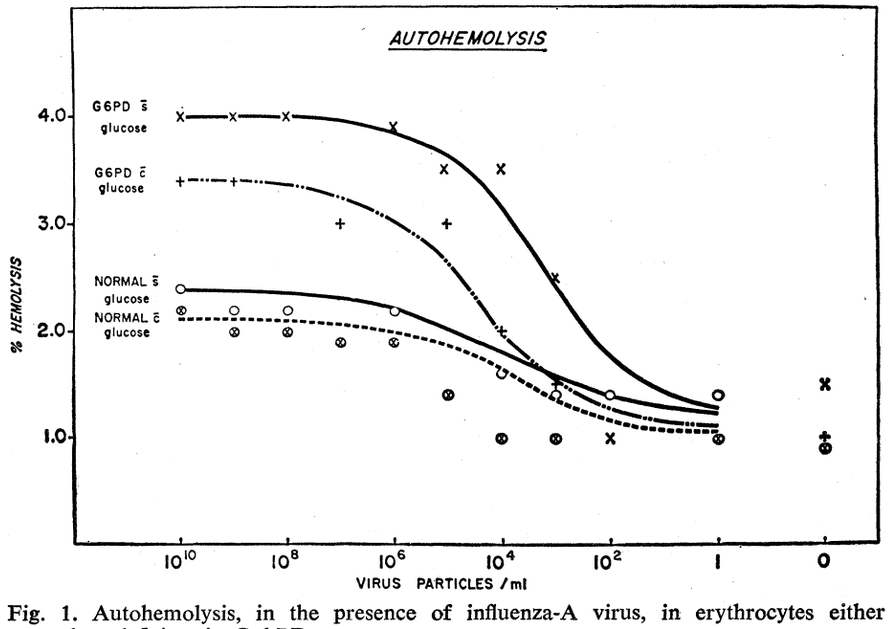

Fig. 1. Autohemolysis, in the presence of influenza-A virus, in erythrocytes either normal or deficient in G-6-PD.

for the decarboxylation of glucose, the production of $C^{14}O_2$ represents the minimal rate of metabolism by way of this pathway.

Red cells were washed three times, the buffy coat was removed, and they were resuspended in a buffered saline medium containing 5 percent albumin and incubated with specifically labeled glucose-C^{14} plus carrier glucose in a final concentration of 200 mg/100 ml. Incubations lasted 4 hours and $C^{14}O_2$ was then recovered and counted. Glucose consumption in duplicate flasks was measured by the glucose oxidase method (*6*). Final results (Table 1) were calculations of micromoles of glucose consumed and CO_2 produced, and percentages of glucose metabolized by way of the hexose monophosphate shunt.

The fact that in red cells deficient in G-6-PD, from two different individuals, hexose monophosphate shunt activity was much lower than in red cells from normal individuals reemphasized the role of G-6-PD in shunt metabolism. In the presence of virus, red cells from such deficient individuals exhibited no increase in hexose monophosphate shunt metabolism. In contrast, red cells from normal individuals, when exposed to viruses, showed a moderate but consistent increase in $C^{14}O_2$ production, reflecting an increase in hexose monophosphate shunt activity. Inactivated virus again failed to elicit the typical increase in CO_2 production in normal cells.

It was therefore concluded that the presence of virus was in some manner associated with an increase in hexose monophosphate shunt activity in normal cells. In red cells severely deficient in G-6-PD, such an increase in shunt activity was not possible; their exposure to virus particles led to an increase in Heinz bodies and eventual hemolysis.

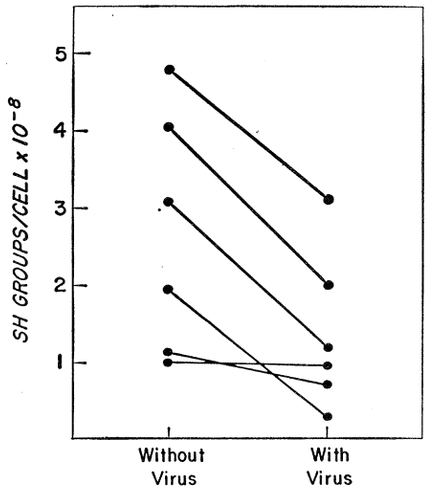

Fig. 2. Total titratable sulfhydryl groups in erythrocytes.

Since red-cell sulfhydryl groups are thought to play an important role in the regulation of hexose monophosphate shunt activity and in the maintenance of red-cell integrity (*7*), total red-cell sulfhydryl groups were measured by a spectrophotometric determination (*8*). In the presence of relatively high concentrations of influenza virus, total titrable red-cell sulfhydryl groups were markedly decreased in four of six normal samples ($P < .01$) (Fig. 2). Virus-induced stimulation of hexose monophosphate shunt activity was unaffected by the addition of *p*-chloromercuribenzoate at 5 μmole/ml, but was completely abolished by addition of *N*-ethylmaleimide at 2.5 μmole/ml (Table 1). Both inhibitors react specifically with sulfhydryl groups: *p*-chloromercuribenzoate reacts only with those on the membrane, while *N*-ethylmaleimide enters the cell freely and is bound to the intracellular sulfhydryl groups (*7*). This series of experiments suggests that the virus particles interact with red-cell sulfhydryl groups, and that this interaction is directly related to the stimulation of the hexose monophosphate shunt.

In the normal red cell, oxidation of sulfhydryl groups activates a series of reparative mechanisms dependent on the presence of reduced glutathione (*7*). The oxidation of glutathione by such reparative mechanisms in turn results in activation of hexose monophosphate shunt. The repair of oxidative damage to red-cell sulfhydryl groups is thus thought to be closely linked to a functioning hexose monophosphate shunt. In erythrocytes in which compensatory increases of hexose monophosphate shunt activity is not possible (that is, in the presence of a marked deficiency of G-6-PD), an increased rate of reduction of sulfhydryl groups is not possible, and the red cell appears incapable of protecting itself against "oxidative" damage.

The presence of increased numbers of Heinz bodies in such cells is a reflection of damage not only to hemoglobin but also to cellular proteins, including those comprising the membrane structure. Precipitation of the cellular proteins, as evidenced by the presence of Heinz bodies, eventually leads to premature destruction of these cells (*9*).

These observations are of interest not only in clarifying a possible mechanism in the hemolysis of cells deficient in G-6-PD, but also in further delineating the interaction of virus particles and intact cells. This interaction may represent a mechanism for the damage of cells, by viruses, quite distinct from the well-known action on nucleic acid metabolism and function (*10*).

Table 1. Hexose monophosphate (HMP) shunt activity in erythrocytes. Washed red cells were suspended in a medium consisting of buffered saline (*p*H 7.40), 5 percent human serum albumin, glucose at 200 mg/100 ml, and glucose-1-C^{14} at 1 μc/ml. The suspension was gassed with 5 percent CO_2 in air and incubated for 2 to 4 hours at 37°C in a Dubnoff metabolic shaking incubator. At the conclusion of the experiment the cell suspension was acidified with 1*N* H_2SO_4, and the $C^{14}O_2$ was collected into 0.2 ml of NCS added through a serum cap into the central well of the flask. Glucose consumption was measured in duplicate flasks by the glucose oxidase method (*6*). Abbreviations: PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide.

Red cells	Micromoles per hour, per milliliter of red cells		Glucose metabolized by way of HMP shunt (%)
	Glucose consumption	CO_2 production	
Normal (*10*)	2.72 ± 0.67	0.103 ± 0.039	3.88 ± 1.22
Normal + virus (*5*)	3.00 ± 1.09*	.150 ± .029†	5.59 ± 0.97†
Plus PCMB	2.60	.100	3.84
Plus PCMB + virus	2.62	.153	5.83
Plus NEM	2.58	.033	1.27
Plus NEM + virus	2.87	.031	1.08
Deficient in G-6-PD	3.21, 6.40	.034, .69	1.05, 1.08
Deficient in G-6-PD, + virus	3.40, 6.88	.015, .70	0.44, 1.01

* Difference not significant. † $P < .05$.

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References and Notes

1. E. R. Burka, A. Weaver III, P. A. Marks, *Ann. Intern. Med.* **64**, 817 (1966); C. Hersko and P. A. Vardy, *Brit. Med. J.* **1967-I**, 214 (1967); G. Salen, F. Goldstein, F. Hauroni, C. W. Wirts, *Ann. Intern. Med.* **65**, 1210 (1966).

2. Influenza-A (PR8) virus obtained by courtesy of Te-Wen Chang, Infectious Disease Service, New England Medical Center Hospitals, and Department of Medicine, Tufts University School of Medicine. Virus harvested in allantoic fluid from chick embryos.
3. W. H. Crosby and F. W. Furth, *Blood* **11**, 380 (1956).
4. J. V. Dacie, *Acta Haematol.* **31**, 177 (1964).
5. H. S. Jacob and J. H. Jandl, *J. Clin. Invest.* **43**, 1704 (1964).
6. A. S. Huggett and D. A. Nixon, *Lancet* **1957-II**, 368 (1957).
7. H. S. Jacob and J. H. Jandl, *J. Biol. Chem.* **241**, 4243 (1966); *J. Clin. Invest.* **41**, 779 (1962); *ibid.*, p. 1514.
8. A. Szeinberg and L. Clejan, *Biochem. Biophys. Acta* **93**, 564 (1964).
9. R. A. Rifkind, *Blood* **26**, 433 (1965).
10. The work was performed under a suballocation from PHS grant SO1-FRO-5598-03. One of us (T.F.N.) is an established investigator of the American Heart Association. We thank Paula Saltman for technical assistance.

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DNA Content of Neurons in the Cat Hippocampus

Abstract. *The DNA content of individual cell nuclei of cat hippocampus has been measured by means of a scanning cytophotometric technique. The pyramidal cells are tetraploid, whereas the remaining cell types, which include glial cells, interneurons, and the granule cells of the dentate gyrus, are diploid. Nuclei of tetraploid pyramidal cells are significantly larger than those of diploid granule cells.*

The finding of tetraploid amounts of DNA (*1*) in normal human (*2*) and rat (*3*) cerebellar Purkinje cells and in autonomic ganglion cells (*4*) has extended knowledge of normal polyploid states in mammals to include the nervous system. The extent of this phenomenon in the nervous system is unknown.

Three cats (2.5 to 3.7 kg) were anesthetized with pentobarbital sodium (25 mg per kilogram of body weight) injected intraperitoneally and perfused through the left ventricle with Ringer solution. The animals were then perfused with a mixture of glacial acetic acid and 95 percent ethanol (1:3). Brain and spinal cord were removed, and blocks were processed in the usual manner for paraffin embedding.

Sections were cut at 22 μ for measurement of hippocampal pyramidal cell nuclei and at 13 μ for measurement of dentate granule cell nuclei. They were stained by the Feulgen reaction (*5*). Absorption of monochromatic light (550 ± 15 nm) by single nuclei was measured with a Barr and Stroud integrating microdensitometer (*6*). Results in arbitrary units represent total nuclear DNA content.

Two cell populations within the hippocampus are easily identifiable morphologically and functionally. The dentate gyrus, consisting of tightly packed granule cells, forms the primary receptive area (*7*). The pyramidal cell layer is composed of large, loosely packed cells and forms the main projection pathway from the hippocampus (*8*). In addition, there are various interneurons and glial cells (*9*).

Figure 1 shows the DNA content in the various cell types of the hippocampus. Glial cells and interneurons form a single population having diploid amounts of DNA (Fig. 1A). In order to establish the tetraploid content of DNA, two diploid glial or interneuron nuclei were measured within the same field, the same conditions of measurement applied to pyramidal cell nuclei being used (Fig. 1A).

The DNA values for granule cells of the dentate gyrus are diploid (Fig. 1, A and B). The pyramidal cell nuclei (Fig. 1B) have a total DNA content significantly higher ($P < .005$) than that for the other cell types measured, comparable to that amount from two glial or interneuron nuclei in the same field (Fig. 1, A and B).

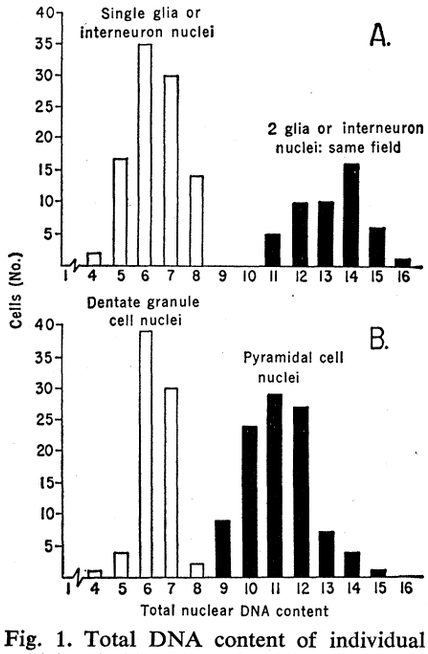

Fig. 1. Total DNA content of individual nuclei of hippocampal cells. Ordinates represent number of cells; abscissas represent the total nuclear DNA content in arbitrary units. (A) Glial and interneuron nuclei (□); 98 nuclei; DNA content, 6.4 ± 0.10 (average ± standard error). Two glial or interneuron nuclei in the same field, measured together (■); 48 nuclei; DNA content, 13.2 ± 0.20. (B) Dentate granule cells (□); 76 nuclei; DNA content, 6.4 ± 0.06. Pyramidal cell nuclei (■); 101 nuclei; DNA content, 11.2 ± 0.10. Data represent pooled values from three animals.

Sections (22 μ) from one of the three animals were treated for 23 hours with deoxyribonuclease before Feulgen staining. Following digestion, measurements of eight pyramidal cell nuclei averaged less than 1 unit, an indication that only a minimum contribution of nonspecific absorption is due to material other than DNA.

Associated with their higher DNA content, 75 pyramidal cell nuclei measured 14.8 ± 1.5 μ (mean ± S.D.) in diameter. Seventy-five dentate granule cell nuclei measured 10.3 ± 2.6 μ in diameter. This difference in size is highly significant ($P<.001$).

Of the cell types in the hippocampus, the pyramidal cells are unique in having larger nuclei with a higher nuclear DNA content. In light of the measurements on two small nuclei in the same field, the most logical interpretation of these data is that the pyramidal neurons are tetraploid. Thus, tetraploidy within the higher integrative centers of the nervous system is not an isolated phenomenon of Purkinje cells. Exploration of the extent of the phenomenon and its correlation with histochemical and physiological data on the cell types involved may give some clue to its functional significance in the nervous system.

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References and Notes

1. "Polyploidy," "tetraploidy," and so forth, as used in this paper refer only to the amount of DNA in the cell nucleus, without implying a doubling of chromosomes rather than chromatids.
2. L. W. Lapham, *Excerpta Med. Int. Congr. Ser. 100* (1965), p. 445; L. W. Lapham, *Science* **159**, 310 (1968).
3. W. Sandritter, V. Novakova, J. Pilny, G. Kiefer, *Z. Zellforsch. Mikroskop. Anat.* **80**, 145 (1967); R. D. Lentz and L. W. Lapham, in preparation.
4. A. A. Kusch and V. N. Yarygin, *Tsitologiya* **7**, 228 (1965).
5. C. Leuchtenberger, in *General Cytochemical Methods*, J. F. Danielli, Ed. (Academic Press, New York, 1958), vol. 1, p. 219.
6. E. M. Deeley, *J. Sci. Instrum.* **32**, 263 (1955).
7. R. H. Laatsch and W. M. Cowan, *J. Comp. Neurol.* **128**, 359 (1966).
8. E. R. Kandel, W. A. Spencer, F. J. Brinley, *J. Neurophysiol.* **24**, 225 (1961).
9. R. Lorente de Nó, *J. Psychol. Neurol.* **46**, 113 (1934).
10. Supported in part by PHS training grant 5T1GM 133-11 and PHS research grant NB07100-01.

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