The Effect of the Nitrite Ion on Intact Human Erythrocytes

By JOHN D. HARLEY AND HELEN ROBIN

THE CHANGES which result when intact erythrocytes are exposed to the nitrite ion have been studied for almost a century. Such exposure has been found to cause the oxidation of oxyhemoglobin to methemoglobin, and of reduced glutathione (GSH) to oxidized glutathione (GSSG). In addition, erythrocytes treated with nitrite have been widely used to investigate the factors involved in the reduction of methemoglobin.

Despite the breadth of these investigations, certain inconsistencies are still apparent. The results of experiments with erythrocytes from various species have been interpreted to indicate that GSH protects against enzyme inactivation, spontaneous hemolysis, choleglobin formation and Heinz body formation. Nitrite-induced GSH depletion might therefore be expected to lead to these changes in vitro, and to excessive hemolysis in vivo. However, the rate of spontaneous hemolysis has been found to be unaffected by the addition of nitrite, the evidence as to Heinz body formation is conflicting, and erythrocyte survival in normal rats has been shown to be unchanged by the administration of nitrite.

Previous investigations as to the effect of nitrite on the GSH content of intact erythrocytes have been made in the absence of glucose or other source of cellular energy. The present work was designed to study the mode of action of the nitrite ion on intact human erythrocytes, both in the presence and absence of adequate substrate and of a normal pentose phosphate pathway.

MATERIALS AND METHODS

Fresh, heparinized, venous blood from normal and glucose-6-phosphate dehydrogenase (G-6-PD)-deficient, adult, Caucasian males was used as a source of erythrocytes. The G-6-PD activity in the enzyme-deficient subjects, assayed by Zinkham's modification of the method of Glaser and Brown, was less than 5 units per 100 ml. of erythrocytes. The blood was centrifuged for 10 minutes at 2100 g and the supernatant plasma and theuffy coat discarded. The erythrocytes were then washed three times with 30 volumes of the appropriate buffer, and resuspended to a packed cell volume of approximately 50 in this buffer.

Depending on the substrate requirements, Krebs-Ringer phosphate buffer, pH 7.4, isotonic glucose-buffer or isotonic lactate-buffer was used. The latter buffers were made up of Krebs-Ringer phosphate buffer, pH 7.4, in which isotonicity had been re-established after the addition of glucose or lactate to a final concentration of 12 mM per liter. In each experiment, the erythrocytes were washed with the appropriate buffer, which was then used throughout.

Test and control incubation mixtures were prepared as described previously, with the following exceptions; that sodium nitrite was used as the test substance, that the

From the Children's Medical Research Foundation, Royal Alexandra Hospital for Children, Sydney, Australia.

Submitted Feb. 6, 1962; accepted for publication July 3, 1962.
buffers described above were used, that larger volumes of the mixtures were prepared, and that the incubation flasks were only shaken at hourly intervals. The initial concentration of hemoglobin in the incubation mixtures was always between 0.45 and 0.50 mM per liter. As required, samples were removed from the test and control mixtures and the following determinations made.

Concentrations of methemoglobin, oxyhemoglobin and "intact" hemoglobin (methemoglobin plus oxyhemoglobin) were determined and expressed as previously described, with the variation that the erythrocytes were washed three times with 30 volumes of Krebs-Ringer phosphate buffer, pH 7.4, before being hemolyzed.

The GSH content was determined by the method of Grunert and Phillips, with the precautions recommended by Flanagan et al. At the commencement, and as required during the period of incubation, 15 ml. samples were taken from the test and control incubation mixtures, centrifuged, and the supernatant fluid aspirated. The cells were then washed four times with 30 volumes of Krebs-Ringer phosphate buffer, pH 7.4, hemolyzed by the addition of distilled water, and the GSH content determined as described above. The decrease in the GSH content of the test and control incubation mixtures was expressed as a per cent of the GSH content of the control incubation mixture at the commencement of the period of incubation.

The G-6-PD activity was determined by Zinkham’s modification of the method of Glaser and Brown. As required, 10 ml. samples were removed from the test and control incubation mixtures, and the supernatant fluid aspirated after centrifugation at 4 C. The cells were then washed three times with 30 volumes of cold isotonic saline, hemolyzed, and assayed for G-6-PD activity. Other than centrifugation as recommended by Zinkham, no precautions for the complete removal of stromata from hemolysates were employed. The G-6-PD activity in the test mixture was expressed as a per cent of the G-6-PD activity in a sample simultaneously assayed from the control incubation mixture.

The fragility to hypotonic saline was determined as described previously. The per cent spontaneous hemolysis was calculated by expressing the optical density at wave length 540 m\(\mu\) of the supernatant fluid as a per cent of the optical density at wave length 540 m\(\mu\) of the hemolyzed whole sample. Erythrocytes were stained and examined for the presence of Heinz bodies as already described.

**Results**

Preliminary experiments showed that the technics used for washing erythrocytes after incubation with nitrite were adequate, no significant difference in results being produced by further washing. Of the various determinations, the GSH content was found to be most sensitive to the presence of residual traces of nitrite; an experiment illustrating this sensitivity will be presented in the course of the results.

**Effect of Brief Incubation with Nitrite**

*Methemoglobin formation and GSH depletion:* Normal erythrocytes suspended in glucose-buffer were incubated for 4 hours with nitrite, in molar ratios to heme ranging from 64:1 to 1:4. At intervals, the degrees of methemoglobin formation and of GSH depletion were measured. From figure 1 it may be seen that increasing concentrations of nitrite caused increasing degrees of methemoglobin formation, culminating in virtually complete conversion of oxyhemoglobin to methemoglobin with a molar ratio of nitrite to heme of 16:1. In addition, methemoglobin production was found to be almost maximal after 15 minutes with the higher concentrations, but increased slowly over the succeeding period of incubation with the lower concentrations of
Fig. 1.—The degrees of methemoglobin formation in normal erythrocytes, suspended in glucose-buffer and incubated for 4 hours with varying concentrations of nitrite. The ratios opposite each curve represent the initial molar ratios of nitrite:heme.

nitrite. After 4 hours, the amount of methemoglobin formed by each lower concentration of nitrite was considerably less than would be expected to result from a stoichiometric reaction between nitrite and heme.

Despite the high degrees of methemoglobin formation, no significant decrease was found in the GSH content of either test or control incubation mixtures, even after 4 hours incubation with nitrite in a molar ratio to heme of 64:1.

To investigate the effect of impaired activity of the pentose phosphate pathway, normal and G-6-PD-deficient erythrocytes were incubated for 1 hour, under varying experimental conditions, with different concentrations of nitrite (fig. 2). From figure 2A it may be seen again that increasing concentrations of nitrite caused increasing degrees of methemoglobin formation, without GSH depletion, in normal cells with glucose as substrate. In the absence of substrate, however, increasing concentrations of nitrite were found to produce increasing degrees of GSH depletion (fig. 2B). The addition of lactate failed to prevent loss of GSH in erythrocytes exposed to nitrite (fig. 2C). Lastly, figure 2D shows that nitrite caused depletion of GSH in G-6-PD-deficient erythrocytes, even in the presence of glucose as substrate. Despite this varying effect on GSH depletion, no striking differences were observed between the degrees of methemoglobin formation produced by each concentration of nitrite under these four experimental conditions (fig. 2).

Even in the absence of nitrite, it may be seen from figures 2B, 2C and 2D that some loss of GSH occurred under circumstances which led to impaired activity of the pentose phosphate pathway. To investigate the effect of time of incubation, these experiments were repeated, with the modification that two concentrations of nitrite were used over a period of 4 hours (table 1). Firstly, it was observed that nitrite in high concentration caused rapid loss of GSH, with more than 85 per cent depletion after 15 minutes incubation. Secondly, in the absence of nitrite, the GSH concentration decreased progressively in normal cells incubated without glucose, but remained stable after an initial fall in G-6-PD-deficient cells with glucose as substrate. Thirdly, nitrite in low concentration appeared to exert a continuing effect in accelerating the proc-
Fig. 2.—Results of four separate experiments as to the degrees of methemoglobin formation and of GSH depletion in erythrocytes incubated for 1 hour with varying concentrations of nitrite. (A) Normal cells, glucose substrate. (B) Normal cells, no substrate. (C) Normal cells, lactate substrate. (D) G-6-PD-deficient cells, glucose substrate.

ness of GSH depletion. Lastly, the degree of methemoglobin formation in the presence of nitrite consistently exceeded the degree of GSH depletion, even though loss of GSH occurred without accumulation of methemoglobin in the absence of nitrite.

It appeared from these various experiments, therefore, that nitrite oxidizes oxyhemoglobin in preference to GSH, which is depleted by nitrite only in situations which lead to impaired activity of the pentose phosphate pathway.

Effect of washing on the determined degree of GSH depletion in erythrocytes incubated with nitrite: The results of early experiments, with inadequate washing of erythrocytes after incubation with nitrite, had suggested that high degrees of GSH depletion may result from exposure to nitrite, even in the presence of glucose. To determine the effect of washing, the GSH content of erythrocytes incubated in glucose-buffer with a high concentration of nitrite was measured after from none to six washes with 30 volumes of Krebs-Ringer phosphate buffer. From table 2 it may be seen that evidence of significant
Table 1.—Effect of Nitrite on the Degrees of GSH Depletion and of Methemoglobin Formation in Normal and G-6-PD-deficient Erythrocytes Incubated for 4 Hours in the Presence and Absence of Glucose or Lactate

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Substrate</th>
<th>Molar Ratio of Nitrite: Heme</th>
<th>GSH Depletion Per Cent</th>
<th>Methemoglobin Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 min.</td>
<td>1 hr.</td>
</tr>
<tr>
<td>Normal</td>
<td>—</td>
<td>64:1</td>
<td>92</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>4:1</td>
<td>*</td>
<td>43</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>1:∞</td>
<td>*</td>
<td>15</td>
<td>39</td>
</tr>
<tr>
<td>Normal lactate</td>
<td></td>
<td>64:1</td>
<td>88</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>4:1</td>
<td>*</td>
<td>25</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>1:∞</td>
<td>*</td>
<td>19</td>
<td>45</td>
</tr>
<tr>
<td>G-6-PD-deficient</td>
<td>glucose</td>
<td>64:1</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>4:1</td>
<td>*</td>
<td>48</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>1:∞</td>
<td>*</td>
<td>19</td>
<td>20</td>
</tr>
</tbody>
</table>

*Not determined.

Table 2.—Effect of Varying Numbers of Washes with 30 Volumes of Krebs-Ringer Phosphate Buffer on the Determined Degree of GSH Depletion in Erythrocytes Incubated for 1 Hour in Glucose-Buffer, with Nitrite in a Molar Ratio to Heme of 64:1

<table>
<thead>
<tr>
<th>No. of Washes</th>
<th>Determined GSH Depletion Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98</td>
</tr>
<tr>
<td>1</td>
<td>87</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

degrees of GSH depletion was observed with less than three, but not with three or more washes. These results were taken as evidence that residual nitrite resulting from inadequate washing may either oxidize GSH directly in hemolysates, or else interfere with the nitroprusside reaction for GSH. In either event, adequate washing would appear essential in any investigation as to the effect of the nitrite ion on intact erythrocytes.

Effect of nitrite on the G-6-PD activity of erythrocytes incubated in glucose-buffer: To investigate the effect of nitrite on one representative -SH enzyme, the G-6-PD activity of normal erythrocytes suspended in glucose-buffer was assayed at intervals during periods of incubation in the presence and absence of nitrite. Over a 4-hour incubation, slight decay in enzyme activity was found in the absence of nitrite, and no increase in this rate of decay was produced by nitrite in molar ratios to heme of 4:1 or less. With higher concentrations of nitrite, however, some evidence of an increased rate of G-6-PD inactivation was obtained. For example, in 10 experiments involving incubation of erythrocytes from normal subjects for 1 hour with nitrite in a molar ratio to heme of 16:1, the mean G-6-PD activity of the test mixtures was found to be 92 per cent of the mean activity of the control mixtures, with a range of 82 to 100
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Table 3.—Effect of Various Concentrations of Nitrite on Heinz Body Production, "Intact" Hemoglobin Destruction, and Osmotic Fragility in Normal Erythrocytes Incubated for 24 Hours, Both with and without Glucose as Substrate

<table>
<thead>
<tr>
<th>Molar Ratio of Nitrite: Heme</th>
<th>No Substrate</th>
<th>Glucose Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heinz body production</td>
<td>&quot;Intact&quot; hemoglobin destruction per cent</td>
</tr>
<tr>
<td>64:1</td>
<td>+++</td>
<td>12</td>
</tr>
<tr>
<td>16:1</td>
<td>++</td>
<td>6</td>
</tr>
<tr>
<td>4:1</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>1:1</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>1:4</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>1:∞</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

The degree of Heinz body formation was assessed arbitrarily.

per cent and a standard deviation of 6 per cent. Five experiments, in which normal erythrocytes were incubated for 4 hours with nitrite in a molar ratio to heme of 64:1, yielded G-6-PD activities in the test mixtures of 68, 77, 83, 88 and 100 per cent of those simultaneously obtained in the control incubation mixtures.

Osmotic fragility and Heinz body studies: Normal erythrocytes were incubated for 4 hours in glucose-buffer with nitrite, in molar ratios to heme ranging from 64:1 to 1:4. Negligible degrees of hemolysis in 0.5 per cent saline, "intact" hemoglobin destruction or Heinz body production were observed.

This experiment was next repeated, using cells suspended in Krebs-Ringer buffer without added glucose. Again, insignificant hemolysis in 0.5 per cent saline was present after 4 hours incubation. However, quite appreciable degrees of Heinz body formation were found with the higher concentrations of nitrite. Thus, with molar ratios of nitrite to heme of 64:1 and 16:1, at least one small but clearly defined Heinz body was found in almost every cell, together with slight but appreciable "intact" hemoglobin destruction in hemolysates. These changes were not observed with lower concentrations of nitrite.

Effect of Prolonged Incubation with Nitrite

Studies were conducted to investigate the gross changes in erythrocytes exposed for long periods, under aseptic conditions, to high concentrations of the nitrite ion. In these prolonged experiments, the flasks were loosely plugged with cotton wool and shaken only at 12-hour intervals.

Initially, normal erythrocytes suspended in Krebs-Ringer buffer without glucose were incubated at 37 C. for 24 hours with varying concentrations of nitrite. From table 3 it may be seen that increased rates of Heinz body production, "intact" hemoglobin destruction, and development of osmotic hyperfragility were produced by the higher, but not by the lower, concentrations of nitrite. These experiments were repeated, using normal erythrocytes suspended in glucose-buffer. Table 3 shows that the higher concentrations of nitrite
again caused Heinz body production, "intact" hemoglobin destruction, and decreased osmotic resistance, but that these effects of nitrite were considerably less in degree than in the absence of glucose.

The result of more prolonged incubation was next investigated. Normal erythrocytes suspended in glucose-buffer were incubated at 37 C. for 5 days, both with and without nitrite in a molar ratio to heme of 64:1. Adequate substrate amounts of glucose were maintained in both the test and control incubation mixtures throughout the periods of incubation.

Results typical of the changes in osmotic fragility and spontaneous hemolysis are shown in figure 3. Hyperfragility to hypotonic saline occurred earlier and increased more rapidly in mixtures containing nitrite. Spontaneous hemolysis was slight in the absence of nitrite, even after 5 days incubation. In contrast, spontaneous hemolysis was apparent in mixtures containing nitrite after 3 days, and was found to increase rapidly after 4 days incubation.

Studies were also made as to Heinz body production and "intact" hemoglobin destruction. After 5 days, negligible changes were seen in cells incubated with glucose in the absence of nitrite. However, gradual changes occurred in those mixtures to which nitrite was added. After 24 hours, as noted
above, slight Heinz body formation and "intact" hemoglobin destruction were found. These changes then increased progressively, so that many Heinz bodies were present in each cell after 4 days. By the 5th day, large amounts of deeply staining material were seen, not only in relation to cells and cell ghosts, but in aggregates of spherical bodies in the extracellular medium. Concomitant with the formation of Heinz bodies, increasing turbidity and "intact" hemoglobin destruction were found in hemolysates. After prolonged centrifugation the hemolysates became clear; the proportional reduction in optical density at wave length 620 m\(\mu\), produced in these solutions by the addition of cyanide, decreased throughout the period of incubation. These findings were taken to indicate the formation, in addition to Heinz bodies, of those soluble degradation products of hemoglobin whose heightened absorption in red light is not abolished by cyanide.

The prolonged experiments would thus appear to provide evidence of a late, destructive effect exerted by the nitrite ion on hemoglobin and the cell membrane.

**Discussion**

The present work has shown that the nitrite ion, even in high concentration, does not cause loss of GSH in normal erythrocytes incubated with glucose as substrate. In the absence of either adequate substrate or a normal pentose phosphate pathway, however, incubation with nitrite in low concentration was found to result in significant degrees of GSH depletion, and in high concentration to produce rapid loss of GSH. It would appear, therefore, that nitrite acts directly to oxidize GSH, which is protected only in the presence of a normal and active pentose phosphate pathway.

The addition of lactate failed to prevent GSH depletion, either in the presence or absence of nitrite. Lactate is oxidized to pyruvate by lactic dehydrogenase, with the generation of reduced diphosphopyridine nucleotide (DPNH).\(^{17}\) Despite previous observations that glutathione reductase in hemolysates may utilize DPNH,\(^{18,19}\) the present results provide evidence that DPNH-dependent pathways play at most a minor part in the reduction of GSSG by the intact human erythrocyte.

Under experimental conditions involving differing levels of activity of the pentose phosphate pathway, no striking variation was observed in the rate of methemoglobin formation by a given concentration of nitrite. With all but the lowest concentration of nitrite, moreover, the degree of GSH depletion was found consistently to fall short of the degree of methemoglobin formation. In view of the molar preponderance of heme over GSH,\(^{20}\) these results suggest that methemoglobin formation buffers the intact erythrocyte against nitrite-induced GSH depletion. They also indicate that an increased rate of GSH oxidation, rather than of methemoglobin formation, may be responsible for the evidence of increased activity of the pentose phosphate pathway which has been elicited in normal erythrocytes exposed to the nitrite ion.\(^{21}\)

In support of these suggestions, reduced triphosphopyridine nucleotide (TPNH) is believed to reduce GSSG by a direct enzymatic mechanism,\(^{22}\) but to mediate the reduction of methemoglobin only in the presence of an arti-
ficial electron carrier such as methylene blue. The rate of oxidation of TPNH almost certainly determines the level of activity of the pentose phosphate pathway. Nitrite might thus activate this pathway by increasing the rate of oxidation of TPNH, acting either indirectly through GSH or directly on TPNH. A metabolic hiatus between methemoglobin and TPNH would help to direct the full activity of the pentose phosphate pathway towards the reduction of GSSG, and the process of non-enzymatic oxidation of GSH by methemoglobin is relatively slow. By absorbing a large part of the oxidation potential of nitrite, heme would thus appear to play a most significant part in the protection of intracellular GSH, and so presumably of the hemoglobin molecule and other cellular components. Some of these interrelations are depicted diagrammatically in figure 4.

The present investigation has also shown that nitrite exerts little effect on the activity in normal erythrocytes of the -SH enzyme, G-6-PD. However, the results do provide some evidence that nitrite in high concentration may cause a slight increase in the rate of decay of G-6-PD activity under conditions which do not lead to GSH depletion. Failing the use of methods which ensure complete removal of stromata from hemolysates, it is possible that any effect of nitrite on G-6-PD activity may be secondary to a primary action on the stromal factor which activates this enzyme.

In the absence of substrate, nitrite appeared to act as a "simple" oxidant, causing increased rates of methemoglobin formation, GSH depletion, oxidative destruction of hemoglobin with Heinz body formation, and loss of osmotic integrity. Even with adequate substrate, high concentrations of nitrite ultimately produced irreversible damage to hemoglobin and the cell membrane.

Fig. 4.—Diagrammatic representation of some mechanisms whereby the nitrite ion acts upon the intact erythrocyte. Continuous lines represent highly probable or certain mechanisms; interrupted lines, possible or minor mechanisms (see text).
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On the basis of previous investigations,\textsuperscript{10,24,27} it seems reasonable to postulate that these effects of nitrite are mainly the result of direct oxidation of the -SH groups of globin and other erythrocytic components. However, deamination by nitrite\textsuperscript{28} may well contribute to the denaturation of cellular proteins. Despite the production of irreversible changes in vitro, the results suggest that the essentially reversible effect of nitrite on erythrocytes in vivo reflects the cellular capacity to maintain adequate levels of GSH. Presumably, the oxidation potential of sublethal doses of nitrite is largely expended in the formation of methemoglobin, which may then be reduced at leisure by DPNH and the Embden-Meyerhof pathway,\textsuperscript{17} leaving the pentose phosphate pathway available for reduction of GSSG. At a cellular level it seems an interesting adaptation, that by temporarily relinquishing oxygen-carrying capacity the erythrocyte may maintain adequate concentrations of GSH and thus protect its various constituents from oxidative destruction. At an organismal level, these mechanisms may be important under evolutionary\textsuperscript{29} or environmental\textsuperscript{100} circumstances which involve intermittent exposure to high concentrations of the nitrite ion.

**Summary**

The mode of action of the nitrite ion on intact human erythrocytes has been investigated under varying experimental conditions. Nitrite appeared to act directly to cause methemoglobin formation and GSH depletion. Evidence was presented to show that such depletion does not occur in the presence of a normal and active pentose phosphate pathway, and to suggest that methemoglobin formation buffers the erythrocyte against nitrite-induced oxidation of GSH.

Both in the presence and absence of glucose, nitrite in high concentration was found to cause increased rates of oxidative destruction of hemoglobin with Heinz body formation, and of loss of osmotic integrity. It was suggested that nitrite acts by "simple" oxidation of the -SH groups of globin and other erythrocytic components, and possibly by causing deamination of cellular proteins.

The results were discussed, with reference to the significance of cellular protective mechanisms under evolutionary or environmental conditions which involve intermittent exposure to high concentrations of the nitrite ion.

**Summario in Interlingua**

Le modo de action de iones de nitrito in intacte erythrocytos human esseva investigate sub varie conditiones experimental. Il pareva que un effecto directe del action de nitrito esseva le causation del formation de methemoglobina e del depletion de reducitate glutathiona (GSH). Es presentate datos e argumentos que monstra que un tal depletion non occurre in le presentia de un normal e active circuito de phosphato de pentosa e que suggere que le formation de methemoglobina age como tampon pro le erythrocytos contra le oxdation de GSH como effecto de nitrito.

Tanto in le presentia como etiam in le absentia de glucosa, il esseva trovate que nitrito in alte concentration causa un accelerate destruction
oxydative de hemoglobina in association con le formation de corposores de Heinz e un accelerate perdita del integritate osmotic. Es suggerite que nitrito age per simple oxydation del gruppos -SH de globina e de altere componentes erythrocytic e possibilemente per causar disamination de proteinas cellular.

Le resultatos del studio es discutite con referentia al signification de mechanismos de protection cellular sub conditiones evolutilional e ambiental que porta con se intermittenute exositiones a iones de nitrito in alte concentration.

ACKNOWLEDGMENTS

Thanks are due to Professor Sir Lorimer Dods, Dr. G. M. Kellerman, Dr. R. D. K. Reye and Dr. R. J. Walsh for helpful and stimulating discussions, to Mr. C. Noble for photographic services, and to Mrs. R. Levitas for expert secretarial assistance.

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John D. Harley, M.B., B.S., M.R.A.C.P., Research Fellow, Children's Medical Research Foundation, Royal Alexandra Hospital for Children, Sydney, Australia.

Helen Robin, B.Sc., Research Assistant, Children's Medical Research Foundation, Royal Alexandra Hospital for Children, Sydney, Australia.
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