CHAPTER - IV

THE ROLE OF IRON IN VIRULENCE OF *Agrobacterium tumefaciens*
IN MICE
Evidence indicates that iron availability in the host system plays a key role in the pathogenesis by bacteria. Iron is required for bacterial growth and in general, microorganisms release iron-chelating compounds or siderophores, which scavenge iron and then deliver the iron load to the microorganism after binding at the membrane receptors (368). According to the siderophore-concept of bacterial virulence, virulent bacteria can grow in animals because they produce large amounts of siderophores which enhance their ability to compete for iron with their hosts (304, 327, 428, 429). Pathogenic microorganisms need iron for growth, and the available iron concentration in tissues of the host may be extremely small. Functional and storage iron is normally found conjugated with proteins and enzymes and hence is more or less inaccessible to invading pathogens. For these reasons it seemed obvious that microbes, endowed with the derepressible high affinity iron transport apparatus, will be able to enhance their iron-scavenging capacity in situ and this will serve as a virulence factor (56, 510). In spite of this it has also been reported by some investigators (127, 279) that there is a poor correlation between the efficiency in siderophore production and the degree of bacterial virulence. The quantities of siderophores which are sufficient to promote growth of bacteria in serum fail to remove iron from transferrin and that the presence of serum-resistant (i.e. virulent) bacteria did not promote the growth of serum-sensitive (i.e. avirulent) bacteria in serum (216, 265, 422). Serum-resistant bacteria can and serum-sensitive bacteria cannot use their stored iron. Siderophores stimulate the growth of serum-sensitive bacteria in serum (277). The ability of siderophores to
stimulate the use of bacterial iron from the outside of bacterial cell suggested an existence of a cell-wall associated factor which, when activated by siderophores, can facilitate the metabolism of bacterial iron; possibility existed that siderophores activate the regulator factor by removing its iron. It has been found that iron bound to bacterial cells can be removed from the cells only with siderophores (277). Study of growth promoting activity of siderophores for serum-exposed bacteria showed that siderophores differ in the efficiency of bacterial stimulation. It has been observed that some heterologous siderophores stimulate the growth of some serum-exposed bacteria more effectively than homologous siderophores (217, 291). This unusual effectiveness of some heterologous siderophores should not be attributed to the power of their chelation of iron but to the accessibility of regulatory protein-bound iron for their activity. This accessibility of iron determines the efficiency with which siderophores stimulate a given species of bacteria; some siderophores reach regulatory protein-iron very effectively, some less effectively, and some cannot reach iron and do not facilitate the use of stored iron.

Concern has been voiced that iron supplementation in early infancy may actually have an adverse effect on resistance to infection (121, 231, 410). The basis for this concern is the fact that microorganisms compete with their host for available iron. It is plausible that iron supplementation by increasing the iron availability both to host and to potential microbial invaders might favour the latter more than the former (34). It has been suggested that hypoferraemia and fever which
commonly accompany bacterial infections may interact as a defence against infection (122, 138, 212). The free serum iron concentration is far below the essential level for bacterial growth due to the serum transferrin which has a very high affinity for iron (410). Thus bacteriostatic activities of serum have been related to the low free serum iron levels (57). In recent years, many studies have shown that in iron treated animals bacterial infections are enhanced (55, 113, 154, 168, 217, 305, 371, 445, 448). This iron overload was suggested to abolish the inhibitory effect of serum on bacterial growth (336).

Ong et al. (297) grew A. tumefaciens on low iron media and isolated a new catechol conjugate of spermidine, named agrobactin. The substance proved to be closely related to a siderophore, compound III, isolated earlier from Paracoccus denitrificans by Tait (373), except that in agrobactin three residues of 2,3-dihydroxy-benzoic acid was present and threonine was shown to occur in an oxazoline ring. Agrobactin (structure I) apparently functions as a siderophore in A. tumefaciens since it was found to correct starvation invoked by the presence of ethylene diamine-di-(O-dihydroxyphenylacetic acid). The three catechol residues plus an oxazoline nitrogen add up to seven possible binding sites which is one more than normally required to satisfy the six co-ordinate ferric ion.

Two hypothesis have been suggested regarding difference in virulence and utilization of iron. The first hypothesis is that the more virulent strain produces more siderophores and therefore has an easier access to iron (139, 327, 428). The second hypothesis is that
there is a lower requirement for external iron supply by the high virulent strain (217). However, the fact remains that a local iron overload in the host system and a simultaneous invasion by pathogenic bacteria corresponds with higher mortality rates (217, 335, 448).

The present study deals with the role and effect of iron made available to a virulent strain of *A. tumefaciens* prior to introduction within the host and present within the host system as an overload in the serum. The problem was dealt with in two ways: firstly, in a given set of animals, iron was introduced within the system and challenged with the bacteria and secondly, the bacteria were incubated in presence of iron and then introduced within the host system. The rate of bacterial growth and the corresponding iron content in the serum and liver were monitored to establish a relationship between iron utilization and bacterial growth and simultaneously, the effect of the activity of several enzymes in mice liver, kidney and brain were measured.
MATERIALS AND METHODS

Organism

Virulent strain of *A. tumefaciens* TIP (Kerr) 14 used in this investigation was maintained at a growth temperature of 28°C on 2% nutrient agar slants of 0.8% Bactotryptone, 0.5% Bactoyeast extract and 0.5% sodium chloride and collected at the mid-log phase of growth around 14 hours after inoculation.

Animal experiments

A] Male Swiss albino mice weighing 22 ± 0.5 gms, maintained on a basal diet (62) were divided into three groups each containing 25 mice. The first group of mice was injected intraperitoneally with ferric citrate at a dose of 200 µg in a volume of 0.2 c.c. per animal. The second group of mice was given the same dose of iron citrate in a volume of 0.1 ml and then after one hour was challenged with an intraperitoneal introduction of *A. tumefaciens* at a concentration of 0.4 X 10^8 cells in a volume of 0.1 ml of physiological saline per animal. The third group of mice was given a direct i.p. injection of *A. tumefaciens* at a dose of 0.4 X 10^8 cells in a volume of 0.2 ml of physiological saline.

Blood was collected by cardiac puncture from the experimental animal in two groups, and monitored for serum iron and bacterial population, the first group contained heparin to prevent coagulation while the second group was without any coagulant so as to
allow the serum to separate. The livers of the experimental animals were also collected for the estimation of iron. Collection from all the three groups was done at time intervals of 2 hours, 4 hours, 24 hours and 48 hours post injection. Six mice were used for each time period.

Iron assay

Serum and liver iron contents were estimated according to the method of Drysdale and Munro (438). Bipyridyl method for the estimation of iron in the ferrous state was chosen, with sodium sulphite as the reducing agent. The iron containing source was made up to 10 c.c. with water, in the presence of sodium sulphite (75 mM), 2,2'-bipyridyl (0.05%) and acetic acid (6% v/v). After heating to 100°C in a water bath for 1 hour, the colour developed was read at 520 nm and the reading was expressed as μg iron/gm tissue or μg iron/ml serum, as required.

Bacterial population

The state of A. tumefaciens multiplication when introduced solely and when introduced with a pre injection of iron was measured by plating of 0.01 c.c. of the blood samples over nutrient agar of minimal media and expressed as colony forming units (CFU)/c.c. of blood.

Animal experiment

B) Male Swiss albino mice weighing 22 gms to 25 gms, maintained on a basal diet (62), were divided into five groups, containing five
mice each. Group 1, served as control and each mice received 0.2 ml pyrogen free saline. Each animal of group 2 received 200 µg of ferric citrate in 0.2 ml physiological saline. Each animal of group 3 received 0.4 x 10^8 cell of A. tumefaciens in 0.2 ml physiological saline. Each animal of group 4 received 0.4 x 10^8 cell of A. tumefaciens incubated previously for 1 hour in 200 µg of ferric citrate, through a volume of 0.2 ml physiological saline. Each animal of group 5 received 200 µg of ferric citrate in a volume of 0.1 ml saline and then after 1 hour 0.4 x 10^8 cells of A. tumefaciens in 0.1 ml saline. All doses were given intraperitoneally. All animals were sacrificed after 18 hours and the liver, kidney and brain were collected and processed for biochemical investigations.

Methods for the preparation of tissue extracts and enzymatic assays

Details of the methods for preparation of tissue extracts and subsequent enzyme assays viz. acid and alkaline phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, glucose-6-phosphatase, Mg^{2+}-ATPase, succinic dehydrogenase, NADH-dehydrogenase, total thiol and glutathione contents, extent of lipid peroxidation, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, superoxide dismutase, catalase, glucose-6-phosphate dehydrogenase, acetylcholinesterase and sodium pyrophosphatase are given in Chapter III.
Determination of protein content

Protein content was estimated according to the method of Gornall et al. (133) and Lowry et al. (248).
Figure 38: Distribution of iron in serum. (o-----o) normal mice given an i.p. injection of ferric citrate.
Fig. 38
Figure 39: Distribution of iron in liver.

(•------•) normal mice given
an i.p. injection of ferric citrate.
Fig. 39

Time (post-injected hour)

Tissue Iron (µg/gm)
Figure 40: Distribution of iron in serum.

(o-o) mice given an i.p. injection of A. tumefaciens.

(●-●) mice given an i.p. injection of ferric citrate prior to introduction of A. tumefaciens.
Fig. 40
Figure 41: Distribution of iron in liver.

(o------o) mice given an i.p. injection of *A. tumefaciens*.

(•------•) mice given an i.p. injection of ferric citrate prior to introduction of *A. tumefaciens*.
Fig. 41

Tissue Iron (µg/gm)

Time (post-injected) hour

2 4 8 16 24 48
Figure 42: Stimulation of *A. tumefaciens* infection in blood by iron.

(○——○) mice given an i.p. injection of *A. tumefaciens*.

(●——●) mice given an i.p. injection of ferric citrate prior to introduction of *A. tumefaciens*. 
Fig. 42
TABLE 12: Effect of *Agrobacterium tumefaciens* on mitochondrial transaminases and NADH dehydrogenase in liver: influence of iron

|                | Glutamate oxaloacetate transaminase (μmoles of pyruvate formed/min/mg of protein) | Glutamate pyruvate transaminase (μmoles of pyruvate formed/min/mg of protein) | NADH dehydrogenase (μmoles of NADH oxidized/min/mg of protein) |
|----------------|----------------------------------------------------------------------------------|--------------------------------------------------------------------------------|-----------------------------------------------------------------
|                | Liver mitochondria                                                               | Liver mitochondria                                                               | Liver mitochondria Kidney mitochondria                             |
| 1] Normal      | 0.156 ± 0.010                                                                     | 0.116 ± 0.006                                                                   | 164.92 ± 13.19 165.42 ± 11.57                                    |
| 2] + Iron      | 0.168 ± 0.015                                                                     | 0.125 ± 0.010                                                                   | 155.29 ± 13.97 178.73 ± 14.29                                    |
| 3] + *Agrobacterium tumefaciens* | 0.216 ± 0.017<sup>a</sup>                                                           | 0.323 ± 0.022<sup>a</sup>                                                           | 187.66 ± 15.01<sup>d</sup> 199.01 ± 13.93<sup>c</sup>             |
| 4] + *Agrobacterium tumefaciens* incubated with iron | 0.230 ± 0.018<sup>a</sup>                                                           | 0.301 ± 0.024<sup>a</sup>                                                           | 189.23 ± 15.13<sup>d</sup> 210.10 ± 16.80<sup>b</sup>             |
| 5] + Iron      + Challenged with *Agrobacterium tumefaciens* after 1 hour    | 0.252 ± 0.017<sup>a</sup>                                                           | 0.333 ± 0.019<sup>a</sup>                                                           | 208.88 ± 14.62<sup>b</sup> 237.95 ± 16.65<sup>a</sup>             |

Each result is expressed as mean value ± S.D. of five experiments, each on a different animal.

Results of groups 2, 3, 4 and 5 have been compared with results of group 1.

Mean value significantly different from the control group <sup>a</sup><sub>p<0.001</sub>, <sup>b</sup><sub>p<0.005</sub>, <sup>c</sup><sub>p<0.01</sub>, <sup>d</sup><sub>p<0.05</sub>.

Results of groups 4 and 5 have been compared with results of group 3.

Mean value significantly different from the control group <sup>1</sup><sub>p<0.001</sub>, <sup>2</sup><sub>p<0.005</sub>, <sup>3</sup><sub>p<0.01</sub>, <sup>4</sup><sub>p<0.05</sub>.
TABLE 13: Effect of Agrobacterium tumefaciens on certain marker enzymes in liver and kidney: influence of iron

<table>
<thead>
<tr>
<th>Glucose-6-phosphatase (µmoles of inorganic P&lt;sub&gt;i&lt;/sub&gt; liberated/hour/mg of protein)</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;-ATPase (µmoles of P&lt;sub&gt;i&lt;/sub&gt; liberated/hour/mg of protein)</th>
<th>Succinic dehydrogenase (µg of K&lt;sub&gt;3&lt;/sub&gt;Fe(CN)&lt;sub&gt;6&lt;/sub&gt; reduced/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver post lysosomal supernatant</td>
<td>Kidney post lysosomal supernatant</td>
<td>Liver mitochondria</td>
</tr>
<tr>
<td>1] Normal</td>
<td>4.38 ± 0.26</td>
<td>3.36 ± 0.23</td>
</tr>
<tr>
<td>2] + Iron</td>
<td>3.96 ± 0.36</td>
<td>3.75 ± 0.26</td>
</tr>
<tr>
<td>3] + Agrobacterium tumefaciens</td>
<td>6.01 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.36 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4] + Agrobacterium tumefaciens incubated with iron</td>
<td>6.42 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.86 ± 0.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5] + Iron + Challenged with Agrobacterium tumefaciens after 1 hour</td>
<td>8.92 ± 0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.17 ± 0.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each result is expressed as mean value ± S.D. of five experiments, each on a different animal.

Results of groups 2, 3, 4 and 5 have been compared with results of group 1. Mean value significantly different from the control group: <sup>a</sup><sub>p</sub><0.001, <sup>b</sup><sub>p</sub><0.005, <sup>c</sup><sub>p</sub><0.01.

Results of groups 4 and 5 have been compared with results of group 3. Mean value significantly different from the control group: <sup>1</sup><sub>p</sub><0.001, <sup>2</sup><sub>p</sub><0.005, <sup>3</sup><sub>p</sub><0.01, <sup>4</sup><sub>p</sub><0.05.
TABLE 14: Effect of *Agrobacterium tumefaciens* on acid phosphatase in liver and kidney: influence of iron

<table>
<thead>
<tr>
<th></th>
<th>Acid phosphatase (μg of p-nitrophenol liberated/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver lysosome</td>
</tr>
<tr>
<td>1] Normal</td>
<td>3.366 ± 0.23</td>
</tr>
<tr>
<td>2] + Iron</td>
<td>4.009 ± 0.32</td>
</tr>
<tr>
<td>3] + <em>Agrobacterium tumefaciens</em></td>
<td>6.055 ± 0.48</td>
</tr>
<tr>
<td>4] + <em>Agrobacterium tumefaciens</em> incubated with iron</td>
<td>6.512 ± 0.45</td>
</tr>
<tr>
<td>5] + Iron + Challenged with <em>Agrobacterium tumefaciens</em> after 1 hour</td>
<td>6.876 ± 0.48</td>
</tr>
</tbody>
</table>

Each result is expressed as mean value ± S.D. of five experiments, each on a different animal.

Results of groups 2, 3, 4 and 5 have been compared with results of group 1.
Mean value significantly different from the control group: $^a_{p<0.001}$, $^b_{p<0.005}$, $^c_{p<0.01}$, $^d_{p<0.05}$.

Results of groups 4 and 5 have been compared with results of group 3.
Mean value significantly different from the control group: $^p_{p<0.001}$, $^q_{p<0.005}$, $^r_{p<0.01}$, $^s_{p<0.05}$. 
TABLE 15: Effect of Agrobacterium tumefaciens on alkaline phosphatase in liver and kidney: influence of iron

<table>
<thead>
<tr>
<th></th>
<th>Liver lysosome</th>
<th>Kidney lysosome</th>
<th>Liver post lysosomal supernatant</th>
<th>Kidney post lysosomal supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1] Normal</td>
<td>2.304 ± 0.138</td>
<td>25.48 ± 1.52</td>
<td>1.678 ± 0.084</td>
<td>3.833 ± 0.26</td>
</tr>
<tr>
<td>2] + Iron</td>
<td>2.520 ± 0.226</td>
<td>22.50 ± 1.80^d</td>
<td>1.778 ± 0.140</td>
<td>3.943 ± 0.27</td>
</tr>
<tr>
<td>3] + Agrobacterium tumefaciens</td>
<td>1.584 ± 0.079^a</td>
<td>18.804 ± 1.50^a</td>
<td>1.923 ± 0.110^c</td>
<td>4.256 ± 0.21^d</td>
</tr>
<tr>
<td>4] + Agrobacterium tumefaciens incubated with iron</td>
<td>1.668 ± 0.116^b</td>
<td>18.652 ± 1.12^a</td>
<td>2.505 ± 0.20^a</td>
<td>4.492 ± 0.26^c</td>
</tr>
<tr>
<td>5] + Iron + Challenged with Agrobacterium tumefaciens after 1 hour</td>
<td>1.259 ± 0.075^a</td>
<td>15.515 ± 1.082^a</td>
<td>2.560 ± 0.17^a</td>
<td>4.829 ± 0.28^a</td>
</tr>
</tbody>
</table>

Each result is expressed as mean value ± S.D. of five experiments, each on a different animal.

Results of groups 2, 3, 4 and 5 have been compared with results of group 1.
Mean value significantly different from the control group: ^a^p<0.001, ^b^p<0.005, ^c^p<0.01, ^d^p<0.05.

Results of groups 4 and 5 have been compared with results of group 3.
Mean value significantly different from the control group: ^1^p<0.001, ^2^p<0.005, ^3^p<0.01, ^4^p<0.05.
TABLE 16: Effect of *Agrobacterium tumefaciens* on certain marker enzymes in brain: influence of iron

<table>
<thead>
<tr>
<th></th>
<th>Alkaline phosphatase (µg of p-nitrophenol liberated/min/mg of protein)</th>
<th>Acid phosphatase (µg of p-nitrophenol liberated/min/mg of protein)</th>
<th>Acetylcholinesterase (µg of acetylcholine hydrolyzed/min/mg of protein)</th>
<th>Sodium pyrophosphatase (µmoles of inorganic P\textsubscript{i} liberated/hour/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain synaptosome</td>
<td>Brain post synaptosomal supernatant</td>
<td>Brain synaptosome</td>
<td>Brain synaptosome</td>
</tr>
<tr>
<td>1) Normal</td>
<td>2.482±0.173</td>
<td>1.306±0.104</td>
<td>3.593±0.287</td>
<td>2.777±0.249</td>
</tr>
<tr>
<td>2) + Iron</td>
<td>1.979±0.178&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.279±0.102</td>
<td>3.618±0.253</td>
<td>2.702±0.216</td>
</tr>
<tr>
<td>3) + <em>Agrobacterium tumefaciens</em></td>
<td>1.076±0.086&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.064±0.085&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.733±0.191&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.196±0.151&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4) + <em>Agrobacterium tumefaciens</em> incubated with iron</td>
<td>1.144±0.091&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.051±0.84&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.964±0.237&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.897±0.151&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5) + Iron + Challenged with <em>Agrobacterium tumefaciens</em> after 1 hour</td>
<td>1.016±0.081&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.889±0.071&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.403±0.192&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.719±0.152</td>
</tr>
</tbody>
</table>

Each result is expressed as mean value ± S.D. of five experiments, each on a different animal.

Results of groups 2, 3, 4 and 5 have been compared with results of group 1. Mean value significantly different from the control group: <sup>a</sup><sub>p</sub><0.001, <sup>b</sup><sub>p</sub><0.005, <sup>c</sup><sub>p</sub><0.01, <sup>d</sup><sub>p</sub><0.05.

Results of groups 4 and 5 have been compared with results of group 3. Mean value significantly different from the control group: <sup>1</sup><sub>p</sub><0.001, <sup>2</sup><sub>p</sub><0.005, <sup>3</sup><sub>p</sub><0.01, <sup>4</sup><sub>p</sub><0.05.
TABLE 17: Effect of Agrobacterium tumefaciens on total thiol, oxidized and reduced glutathione contents in liver: influence of iron

<table>
<thead>
<tr>
<th></th>
<th>Total thiol (µg total thiol/mg of protein content)</th>
<th>Glutathione (reduced) (µg glutathione/mg of protein)</th>
<th>Glutathione (oxidized) (nmoles of GSSG/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole liver homogenate</td>
<td>Whole liver homogenate</td>
<td>Whole liver homogenate</td>
</tr>
<tr>
<td>1) Normal</td>
<td>1.029 ± 0.06</td>
<td>0.94 ± 0.05</td>
<td>2.65 ± 0.15</td>
</tr>
<tr>
<td>2) + Iron</td>
<td>1.014 ± 0.06</td>
<td>1.09 ± 0.08</td>
<td>2.43 ± 0.17</td>
</tr>
<tr>
<td>3) + Agrobacterium tumefaciens</td>
<td>1.019 ± 0.05</td>
<td>1.81 ± 0.14</td>
<td>3.79 ± 0.22</td>
</tr>
<tr>
<td>4) + Agrobacterium tumefaciens incubated with iron</td>
<td>0.918 ± 0.04</td>
<td>1.78 ± 0.14</td>
<td>3.92 ± 0.25</td>
</tr>
<tr>
<td>5) + Iron + Challenged with Agrobacterium tumefaciens after 1 hour</td>
<td>0.881 ± 0.05</td>
<td>1.93 ± 0.15</td>
<td>4.07 ± 0.29</td>
</tr>
</tbody>
</table>

Each result is expressed as mean value ± S.D. of five experiments, each on a different animal.

Results of groups 2, 3, 4 and 5 have been compared with results of group 1.
Mean value significantly different from the control group: a<0.001, b<0.005, c<0.01, d<0.05.

Results of groups 4 and 5 have been compared with results of group 3.
Mean value significantly different from the control group: 1p<0.001, 2p<0.005, 3p<0.01, 4p<0.05.
TABLE 18: Effect of *Agrobacterium tumefaciens* on lipid peroxidation, catalase, and superoxide dismutase in liver: influence of iron

<table>
<thead>
<tr>
<th></th>
<th>Lipid peroxidation (μmol MDA/mg protein)</th>
<th>Catalase (moles of H₂O₂ decomposed/min/mg of protein)</th>
<th>Superoxide dismutase (units*/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole liver homogenate</td>
<td>Liver post microsomal supernatant</td>
<td>Liver post microsomal supernatant</td>
</tr>
<tr>
<td>1] Normal</td>
<td>0.049 ± 0.003</td>
<td>0.2669 ± 0.016</td>
<td>30.26 ± 2.11</td>
</tr>
<tr>
<td>2] + Iron</td>
<td>0.052 ± 0.003</td>
<td>0.2638 ± 0.015</td>
<td>28.03 ± 1.96</td>
</tr>
<tr>
<td>3] + <em>Agrobacterium tumefaciens</em></td>
<td>0.057 ± 0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1831 ± 0.012&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.91 ± 1.55&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4] + <em>Agrobacterium tumefaciens</em> incubated with iron</td>
<td>0.059 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1725 ± 0.0013&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.07 ± 1.56&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>5] + Iron + Challenged with <em>Agrobacterium tumefaciens</em> after 1 hour</td>
<td>0.062 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1649 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.44 ± 1.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each result is expressed as mean value ± S.D. of five experiments, each on a different animal.

Results of groups 2, 3, 4 and 5 have been compared with results of group 1.
Mean value significantly different from the control group <sup>a</sup><sub>p<0.001</sub>, <sup>b</sup><sub>p<0.005</sub>, <sup>c</sup><sub>p<0.01</sub>, <sup>d</sup><sub>p<0.05</sub>.

Results of groups 4 and 5 have been compared with results of group 3.
Mean value significantly different from the control group <sup>1</sup><sub>p<0.001</sub>, <sup>2</sup><sub>p<0.005</sub>, <sup>3</sup><sub>p<0.01</sub>, <sup>4</sup><sub>p<0.05</sub>.

*One unit of enzyme activity is defined as that amount which causes 50% inhibition of pyrogallol oxidation in a reaction volume of 3 ml.
<table>
<thead>
<tr>
<th>Glucose-6-phosphate dehydrogenase (mmoles of NADPH formed/min/mg of protein)</th>
<th>Liver post microsomal supernatant</th>
<th>Glutathione peroxidase (µmoles of NADPH oxidized/min/mg of protein)</th>
<th>Liver post microsomal supernatant</th>
<th>Glutathione-S-transferase (µmoles of product formed/min/mg of protein)</th>
<th>Liver post microsomal supernatant</th>
<th>Glutathione reductase (µmoles of NADPH disappeared/min/mg of protein)</th>
<th>Liver post microsomal supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1] Normal</td>
<td>0.0105 ± 0.007</td>
<td>0.0577 ± 0.004</td>
<td>0.2199 ± 0.017</td>
<td>0.0134 ± 0.008</td>
<td></td>
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</tr>
<tr>
<td>2] + Iron</td>
<td>0.01008 ± 0.0008</td>
<td>0.0590 ± 0.004</td>
<td>0.2560 ± 0.017d</td>
<td>0.0118 ± 0.008d</td>
<td></td>
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</tr>
<tr>
<td>3] + Agrobacterium tumefaciens</td>
<td>0.01508± 0.009a</td>
<td>0.0793 ± 0.005a</td>
<td>0.3120 ± 0.028a</td>
<td>0.0086 ± 0.005a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4] + Agrobacterium tumefaciens incubated with iron</td>
<td>0.0149 ± 0.001a</td>
<td>0.0787 ± 0.006a</td>
<td>0.3164 ± 0.018a</td>
<td>0.0101 ± 0.00006 2a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5] + Iron + Challenged with Agrobacterium tumefaciens after 1 hour</td>
<td>0.01781± 0.0012a</td>
<td>0.1038 ± 0.009a</td>
<td>0.3264 ± 0.022a</td>
<td>0.0064 ± 0.004a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each result is expressed as mean value ± S.D. of five experiments, each on a different animal.

Results of groups 2, 3, 4 and 5 have been compared with results of group 1.
Mean value significantly different from the control group a p<0.001, b p<0.005, c p<0.01, d p<0.05.

Results of groups 4 and 5 have been compared with results of group 3.
Mean value significantly different from the control group 1 p<0.001, 2 p<0.005.
The fate of iron citrate after injection was investigated to study its localisation and distribution with respect to time within the physiological iron pools. Figures 38 and 39 show the passage of iron with time in serum and in the liver. In the blood the iron level rises sharply for 4 hours after introduction of iron and then gradually decreases till 24 hours after which the level becomes constant, while in the liver the iron level gradually rises till 24 hours when it reaches a peak and the liver remains saturated with iron till 48 hours. The fate of iron in the serum and in the liver respectively on injecting mice with (a) *A. tumefaciens* and (b) *A. tumefaciens* with a pre-injection of iron has been shown in Figures 40 and 41. The data shows an overshoot of iron around 4 hours after injection which gradually decreases till 48 hours after injection in serum. While in the liver, the iron concentration gradually rises till 24 hours after which time there is a decline, in the group treated with iron and Agrobacteria. In the former group where there is no local iron overload, there is no usable supply of iron, and the basal iron content in serum and liver remain more or less constant.

In order to compare the state of the bacterial population within the treated mice, a simultaneous monitoring of the microorganisms in blood was made. Figure 42 shows the distribution of *A. tumefaciens* in blood when only the bacteria were injected and when the bacteria were injected with a pre introduction of iron. In the former group of mice, the bacterial count in blood rises till
8 hours after injection indicating that this period of time is required by the microorganisms to enter the circulatory system from the peritoneum, after 8 hours the bacterial count constantly decreases till 48 hours indicating that the bacteria either become non-viable or pass out of the circulatory system into the visceral organs. In the latter group, there is a rapid rise in the bacterial count till 24 hours after injection after which the count decreases until 48 hours.

The rapid bacteremia that occurred after the i.p. injection of A. tumefaciens strain TIP K14 into normal mice, revealed some important aspects of the virulence of this organism. The bacterium must have escaped from phagocytes in the peritoneal cavity, possessed the ability to invade the blood stream from the peritoneal cavity, and obtained sufficient iron for its rapid growth. But as already seen previously the bacteria did not stabilize in the blood, and after 8 hours after injection began to disappear from the blood stream to reach other organs of the viscera. In the normal course of events, administration of parenteral iron, in the form of ferric citrate, led to its rapid reception in the blood stream to reach a peak as soon as within 4 hours after introduction into the peritoneal cavity, wherefrom the level of free iron gradually diminished until 24 hours to reach a basal level. Correspondingly the level of iron in the liver gradually rose until 24 hours post injection. A similar bacterial inoculation with a prior booster of ferric citrate at the same site resulted in massive acceleration in the growth of A. tumefaciens in the blood stream for as long as
24 hours after injection, after which the level decreased though still remained above the control value. It is evident from the data that until 4 hours after introduction of the bacteria, the organisms do not multiply very rapidly, this time corresponds with the time until which there is an iron overload in the blood. The iron level rises in the liver after this time and so also do the bacterial colonies; from 4 hours until 24 hours there is a sharp overshoot in the bacterial count when boostered with a pre injection of iron. Concomitantly, the iron level in the blood rises until 4 hours after which it rapidly falls while in the liver the level rises constantly until 24 hours.

Normal animals when exposed to *A. tumefaciens* show that basal iron level cannot be utilized by the bacteria in the blood, instead there is a slow iron utilisation procedure in the liver as the level of iron gradually decreases until 24 hours after introduction of the bacteria into the host body, after which time the bacterial infection disappear. The studies with the normal mice injected with the bacteria indicate that the infection may have been controlled due to iron limitation whereas iron addition stimulated the infection to a very great extent. The fate of iron after injection was investigated to study the time course of its distribution within the physiological iron pools so that the location of extra iron could be related to its stimulation of infection. Iron entered the circulatory system rapidly after an intraperitoneal injection, with a peak level of serum iron occurring about 4 hours post injection. The serum iron level then rapidly declined to pre injection level by
about 48 hours post injection. Elevated iron levels were detected in the liver which peaked around 24 hours post injection. These data indicate that iron is rapidly removed from the circulation by the reticuloendothelial system and the iron overload of the RES does not alter the ability of *A. tumefaciens* to establish an infection in mice. Such iron deposits in the RES were observed in the liver sinusoids (168). It is also seen that introduction of iron and bacteria at the same site, same time failed to produce any detectable change in infection from that produced in normal mice by the microorganism. This indicates that a high serum iron level was necessary to cause a stimulation of infection. Thus the growth of serum exposed Agrobacterium is facilitated by enriching the serum with iron. Experiments have shown that iron promotes the development of tuberculosis not only in normal but also in immune mice (213, 214). The mechanism by which this iron overload facilitates bacterial multiplication in normal and immune animals is unknown. The growth promoting effect of iron for serum exposed bacteria suggest that the infection promoting activity of iron should not be attributed exclusively to its nutritional value. However, a possibility exists that iron, in addition to being an essential nutritite, also interferes with the activity of some antibacterial and bactericidal systems.

The groups under study are mice treated with (a) iron, (b) *A. tumefaciens*, (c) the same amounts of iron and *A. tumefaciens* injected together after incubation for 1 hour, and (d) iron given as a pre-injection, 1 hour prior to injection of *A. tumefaciens*
at the same site. All four groups have been compared to a normal set of mice and the last two groups have also been compared with the group (b) which received only the bacteria. The status of mitochondrial liver transaminases—glutamate oxaloacetate transaminase and glutamate pyruvate transaminase, have been presented in Table 12. It is seen that only iron administration to normal mice did not cause any radical change in the activities of the two enzymes. The bacteria when injected on being incubated in the same amount of iron caused a sharp rise in the enzymes' activities which remained similar to that when only Agrobacterium was injected. But when iron was pre injected, followed by a bacterial introduction after 1 hour there was a still greater stimulation in the activities of both the transaminases.

A. tumefaciens causes marked liver damage resulting in elevated levels of transaminases in the liver, after bringing about an extensive tissue destruction. A further aggravation of the situation was brought about when iron was pre injected into the host system, owing to the increase in virulence of the microorganisms caused by the presence of free iron in the physiological system.

The status of both the liver and kidney mitochondrial NADH dehydrogenases have been presented in Table 12. Agrobacterium as already seen, causes a rise in the activity of the enzyme in both organs and when introduced into mice which received a pre injection of iron, brought about a severe rise in the enzyme activity especially in the kidney, whereas when injected on being pre incubated in the same dosage failed to cause any significant change from that when
only *A. tumefaciens* was introduced into normal mice. A treatment of an equivalent dose of iron caused a very slight perturbation in the enzyme activity but was not significant enough to register any change.

The effect of *A. tumefaciens*, iron, *A. tumefaciens* incubated in iron and iron challenged 1 hour later with *A. tumefaciens*, on the status of glucose-6-phosphatase in the microsome rich post lysosomal supernatant of liver and kidney, Mg$^{2+}$-ATPase and succinic dehydrogenase in the liver and kidney mitochondria, have been compared in Table 13. It is seen that iron did not cause any noticeable alteration in the status of any of these enzymes. *A. tumefaciens* caused marked elevations in the activities of glucose-6-phosphatase and Mg$^{2+}$-ATPase in the liver and kidney, with a concurrent depression in succinic dehydrogenase activity in liver. *A. tumefaciens* when incubated in iron and then introduced into the host body caused changes very similar to that as observed when only *A. tumefaciens* was injected. But when iron was injected 1 hour before the introduction of the bacteria, the entire situation worsened, leading to a further rise in the activities of glucose-6-phosphatase and Mg$^{2+}$-ATPase and a suppression of liver succinic dehydrogenase. This deterioration was significantly greater from that when only *A. tumefaciens* was injected.

The elevation in the activity of NADH dehydrogenase (Table 12) is parallel to the finding of elevation in the activity of Mg$^{2+}$-ATPase. A rapid hydrolysis of ATP necessitates a rapid generation of ATP and an increase in the requirement of NAD$^+$ leads to a perturbations in
the NADH/NAD<sup>+</sup> ratio. This in turn acts on the availability of
oxaloacetate which has increased owing to raised GOT activity,
causing an inhibition of succinic dehydrogenase activity as
oxaloacetate is a potential suppressor of the enzyme. Thus the
energy metabolism of the host system has been hampered together with
an inhibition of glycolysis owing to raised glucose-6-phosphatase
activity. It is also significant that iron when injected along with
the bacteria could not bring about any appreciable change, but when
injected previously to the bacteria so as to allow a pre loading of
iron in the blood and liver, caused an enhancement of virulence
which is apparent by the noticeable deterioration of the enzymatic
situation in the host system.

Tables 14 and 15 show the status of acid phosphatase and alkaline
phosphatase respectively in the liver and kidney, lysosome and
post lysosomal supernatant. The activity of acid phosphatase
increased in all fractions of both organs with all treatments;
alkaline phosphatase activity decreased in the lysosome and increased
in the post lysosomal supernatant fraction of both organs with all
the treatments. Iron when administered separately did not cause any
appreciable change in any fraction. A very significant aggravation
of the activities of both the phosphatases are seen in all the
fractions of both organs when the mice treated with A. tumefaciens
were given a local boosting of iron prior to bacterial invasion.
This aggravation is absent when bacteria were incubated in iron
outside the host body. This conforms with the suggestion that a
pre load of local iron in the host body causes an enhancement of
bacterial virulence owing to the utilisation of iron by the bacteria for growth and virulence within a host body.

Table 16 depicts the activities of acid and alkaline phosphatases in the synaptosome and post synaptosomal supernatant fraction, acetylcholinesterase in the synaptosome and sodium pyrophosphatase in the post synaptosomal supernatant fraction of brain. The acid and alkaline phosphatases in all the fractions decreased on *A. tumefaciens* treatment. A local increase in the iron level in the system did not very much alter the normal activity of these enzymes, but when later challenged with *A. tumefaciens* the condition of these enzyme activities deteriorated very much. This deterioration was not so great when *Agrobacterium* was injected on being pre incubated with the same concentration of iron. Conversely, the activities of acetylcholinesterase and sodium pyrophosphatase were elevated.

The content of total thiol, oxidized and reduced glutathione in liver are presented in Table 17. The reduced glutathione content was slightly elevated on iron treatment though the oxidized glutathione and total thiol content remained constant. A treatment of *Agrobacterium* caused the glutathione contents to be raised though the thiol content was not perturbed. The glutathione contents remained elevated when treated with *A. tumefaciens* incubated with iron and when an iron overload was challenged with *Agrobacterium*; though these changes were not significantly different from only *Agrobacterium* treated mice. However, in both these two groups of treatment the thiol content decreased considerably.
The status of lipid peroxidation, and activities of catalase and superoxide dismutase have been shown in Table 18 and the status of glutathione reductase, glutathione peroxidase, glutathione-S-transferase, and glucose-6-phosphate dehydrogenase activities have been given in Table 19. Iron treatment caused a slight perturbation in the activities of glutathione-S-transferase and glucose-6-phosphate dehydrogenase, but otherwise does not cause much disturbances elsewhere. A. tumefaciens treatment raised lipid peroxidation together with the activities of glutathione reductase, glutathione peroxidase and glutathione-S-transferase, while causing a depression in the activities of catalase, superoxide dismutase and glucose-6-phosphate dehydrogenase. These perturbations were similar to that when A. tumefaciens was injected on being incubated in iron. But when A. tumefaciens was injected with a pre injection of iron, the deterioration was far greater and was significantly aggravated from that when only A. tumefaciens was injected. A pre treatment of iron to bacterial invasion caused an enhancement of virulence thereby increasing the toxic condition of the system leading to a greater formation of free radical and peroxides by a rapid lipoperoxidation causing a faster generation of GSH to GSSG and a rapid adaptation by the scavenging system of enzymes which was necessary in order to meet the greater demand for the prevention and repair of cellular damage.

The results presented, indicate two aspects regarding the understanding of host-parasite relationships; the first, deals with bacterial virulence and the second with the infection promoting
effect of iron. The most important determinant of bacterial virulence is the ability of bacteria to multiply in animals, which is usually determined by their ability to compete with their host for essential nutrients, suggesting that bacterial nutrition determines the efficiency of bacterial parasitism (56, 215, 409). The best known example of the antibacterial activity because of the nutritional deficiency in animals is the limitation in the availability of iron. The ability of bacteria to acquire iron in animals has been associated with bacterial virulence, and an important role in this acquisition is played by extracellular or outer membrane associated siderophores (127, 327). Iron is necessary for the production of essential enzymes and consequently for the manufacture of factors for bacterial virulence. Virulent bacteria obtain iron from transferrin-iron complexes by a process in which cell wall associated siderophores, lipopolysaccharides and specific receptors for siderophore—iron complexes play a part (216, 225). Some investigators conclude that bacterial growth in serum is promoted by excreted siderophores which remove iron from transferrin and, in some way, deliver it to the bacterial cell, while others believe that siderophores do not remove iron from transferrin but form transferrin-iron-siderophore complexes, which are used much more efficiently by virulent bacteria than by avirulent ones (216, 225). Moreover virulent bacteria grow in serum not because they are prolific in the production of siderophore but because they possess outer membrane associated—siderophores which bind transferrin-iron to bacterial cells (127, 216).
Iron promoted the development and progression of serum exposed *Agrobacterium* in mice, which could well be attributed to the fact that iron promotes the growth of bacteria in mammalian sera by serving as an essential nutrient. The free serum iron concentration of about $10^{-18}$ M (57) in normal serum is far below that required for bacterial growth (410). Although iron may become more available during disease owing to haemorrhage, lysis of erythrocytes, or tissue necrosis, the initial low level of free iron suggests that a bacterial pathogen must employ an iron acquisition mechanism capable of competing with host iron binding proteins during the establishment of infection. *Salmonella typhimurium* (225) and *Neisseria meningitidis* (6) have been found capable of removing iron from transferrin, *in vitro*. Iron administration to host animals has been shown to greatly enhance their susceptibility to experimental bacterial infections (57, 154, 167, 217, 410). Evidences given by Holbein (168) suggest that serum iron rather than the reticulo-endothelial system iron is important in determining the course of infection.