Factors influencing the anaerobic glycolysis of brain and tumour

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The effects of calcium ions, a variety of organic bases and sodium pyruvate on the anaerobic glycolysis of guinea-pig brain and tumour slices have been investigated. The presence of calcium ions exercises a marked stimulation of the conversion of glucose into lactic acid in presence of guinea-pig brain slices when these are bathed in a calcium-free medium at pH 7.5.

This inhibitory effect on glycolytic rate resulting from the absence of calcium is largely overcome by decreasing the pH to 7.0. The optimal stimulating concentration of calcium is 0.004 M. The presence of 0.001 M-sodium pyruvate accelerates anaerobic brain glycolysis very markedly in a calcium-free medium, but only slightly in the presence of an optimal calcium concentration. A number of organic bases (pyrrole, quinoline, p-chloraniline, benzidine, α-naphthylamine, 2:4-lutidene, pyridine, aniline, triethylamine, choline, N-methylpyridine, urea, nicotinamide) accelerate anaerobic brain glycolysis in a calcium-free medium, the concentrations needed for optimal activity varying considerably, e.g. pyrrole, 32 mM; quinoline, 1.6 mM; urea, 64 mM; nicotinamide, 200 mM. The efficiency of the organic bases in replacing calcium for anaerobic brain glycolysis can be, in general, correlated with their dissociation constants.

The effects of the organic bases cannot be explained in terms of DPN-ase inhibitions. Anaerobic glycolysis of tumour slices exhibits little or no calcium dependence and is not affected by the organic bases effective with brain. No additive effects are obtained by combinations of calcium ions, organic bases and sodium pyruvate used at their optimal stimulating concentrations. The results point to a common site of action of all the stimulating substances, or to a common effect on a rate-limiting step in brain anaerobic glycolysis. There seems to be no correlation between the abilities of the organic bases to stimulate glycolysis and their abilities to inhibit DPN-ase. The results of physiological tests show that the organic bases pyrrole, quinoline and p-chloraniline exert neurological effects simulating that of calcium ions in abolishing the spontaneous firing of cat cervical sympathetic ganglia. Unlike calcium ions, however, there was no restoration of sympathetic transmission. The bases act in physiological systems at the same concentrations and with about the same relative potency as in anaerobic glycolysis. It seems that the bases (as well as calcium) establish new membrane equilibria reflected on the one hand by marked neurphysiological activities and on the other by changed glycolytic rates. The suggestion is made that the neurone surface is an important site of glucose metabolism and that the DPN-DPNH ratio there plays a significant rate-limiting role.

INTRODUCTION

It is well known that the metabolism of intact brain tissue (in the form of slices) is greatly influenced by the cation composition of the medium in which the brain tissue is immersed. Ashford & Dixon (1935) showed that the addition of 0.1M-KCl to a Ringer medium containing freshly cut brain cortex slices brought about increased rates of respiration and aerobic glycolysis and a decreased rate of anaerobic glycolysis. Dickens & Greville (1935) made a comprehensive study of the effects of cation concentration and balance on tissue respiration and glycolysis, and pointed out the difficulty of assigning a particular role to a given ion owing to the interaction that occurs in the presence of mixtures of ions. Among a variety of results, they demonstrated the effects of calcium ions in lowering the high (but unsteady) respiratory activity and the high rate of aerobic glycolysis of brain cortex slices in a medium in which sodium was the only cation present. The further addition of potassium ions resulted in an almost complete abolition of aerobic glycolysis, but respiratory activity was increased. The addition of potassium ions at a high concentration (0.1M) greatly increased aerobic glycolysis. It was therefore evident that the effects of potassium on brain aerobic glycolysis are greatly dependent on its concentration.

The rate of anaerobic glycolysis of brain slices was diminished by addition to a sodium medium of a mixture of potassium, calcium and magnesium cations. Dickens & Greville noted the fact that the changes in cationic composition, which produced large changes in brain metabolism, had no such effects on the metabolism of tissues such as kidney cortex, testis, rat yolk sac, retina, Jensen sarcoma and Walker carcinoma 256. The view was advanced that the effects of cations on brain metabolism are due to changes that they induce in membrane permeability.

It should be noted that, although Dickens & Greville (1935) found that no effect on tumour anaerobic glycolysis was produced by the addition of 0.1M-KCl, Lasnitzki (1933) and Lasnitzki & Rosenthal (1933) had earlier stated that the anaerobic glycolysis of rat tumours was increased when K+ was added to a K+, Ca2+-free medium. With Jensen sarcoma, the presence of 2.5 mM-KCl raised the rate of anaerobic glycolysis by 70%, and the further addition of 1.8 mM CaCl₂ raised it by 115%.

Quastel & Wheatley (1937a, b) showed that the anaerobic glycolysis of brain cortex slices was markedly stimulated by the addition of calcium salts to a sodium-potassium medium. The addition of magnesium had a lesser effect in a medium containing sodium, potassium and calcium. They also reported a stimulation of anaerobic glycolysis by the organic bases pyrrole, pyridine and nicotine.

The effect of ammonium ions on tissue glycolysis was studied by Weil-Malherbe (1938). Ammonium chloride (0.033M) produced an effect similar to that of 0.1M-KCl, i.e. it caused a rise in the rate of oxygen uptake and of aerobic glycolysis and an inhibition of the rate of anaerobic glycolysis. Glutamic acid (0.01M) and a few closely related substances were found to inhibit the anaerobic glycolysis of brain slices. The presence of pyruvate reversed the effect of glutamic acid.
Canzanelli, Rogers & Rapport (1942) concluded that calcium and potassium are the only cations controlling respiration. In depressing respiration, calcium apparently acted by inhibiting the stimulating action of potassium. Peiss, Hall & Field (1949) found that the presence of magnesium ions accelerated the anaerobic glycolysis of brain slices.

Recent work on glycolysis in brain homogenates has shown that the ionic composition necessary for the optimal rate of glycolysis differs considerably from that required for optimal glycolysis in brain slices. Racker & Krimsky (1945) found that sodium ions inhibited the anaerobic glycolysis of mouse-brain homogenates. Utter (1950), studying the mechanism of the sodium inhibition of the anaerobic glycolysis of rat-brain homogenate, noted that high rates of glycolysis were obtained when the medium contained mainly potassium or ammonium salts. The addition of sodium salts resulted in a marked inhibition of glycolytic rate and there appeared to be a sodium-potassium antagonism. The inhibition by sodium ions could also be reversed by the addition of hexose diphosphate. This author also studied the effect of sodium ions upon various individual reactions of the glycolytic chain and suggested that there are three interlocked effects—a stimulation of conversion of \( \text{ATP} \) to \( \text{AMP} \), an inhibition of glycolysis by the \( \text{AMP} \) thus formed, and a decreased rate of removal of \( \text{AMP} \) by rephosphorylation.

However, as has already been pointed out, the presence of a high concentration of potassium ions markedly reduces the rate of anaerobic glycolysis in brain cortex slices. It is clear that potassium ions exert different effects on brain glycolysis according to the state of the tissue. The presence of calcium is necessary for high anaerobic glycolysis activity in brain cortex slices, but does not seem to be important for high glycolytic activity in brain homogenates.

A study has been made in the present work of the mechanism of calcium stimulation of anaerobic glycolysis of brain and of the effects of a variety of organic substances which have been found to simulate the effect of calcium. Intact brain slices have been used throughout.

**Materials and methods**

**Preparation of brain slices**

Young adult guinea-pigs were stunned by a blow on the neck, decapitated and the brains removed. The hemispheres were parted down the mid-line, and the mid-brain dissected out and discarded. The hemispheres were then sliced transversely, one at a time, using a McIlwain chopper set to give slices of 300\( \mu \) thickness. Separation of the slices was effected in the saline medium (ice-cold) described below, to which glucose had been added, and only the slices from the middle third of each hemisphere were used. The separated slices were weighed after being drained as much as possible and then placed into Warburg vessels. Approximately 40 mg of slices were added to each vessel.

Dry-weight determinations showed that 100 mg wet weight of slices, prepared under these conditions, were equivalent to approximately 14 mg dry weight.
Tumour tissue

Samples of Novikoff hepatoma grown in rats, and of sarcoma 37 grown in mice or in fertile eggs, were kindly provided by Mr J. A. Kochen of this Institute. These tissues gave unsatisfactory slices with the McIlwain chopper, and were consequently cut in a Stadie-Riggs apparatus.

Estimation of anaerobic glycolysis

Anaerobic glycolysis was estimated by measuring CO₂ evolution from a bicarbonate medium in the Warburg apparatus, at pH 7·5 except where otherwise stated, and at 37-5° C in an atmosphere of nitrogen containing 7 % carbon dioxide. In most of the experiments, the gas mixture was not treated to remove traces of oxygen. When this was necessary, it was done by passing the mixture over heated copper. All determinations were made in duplicate, and controls were included, the media containing slices but no glucose. Slices prepared as described above were highly active and good duplicates of results were almost invariably obtained. Except where otherwise stated, experiments were of 2 h duration, the first 20 min reading being discarded. Estimations were, therefore, made over the 20 to 120 min period, and the glycolytic rate was quite steady for this length of time. Where glycolysis readings are given per 100 mg tissue per hour, the rates are corrected to 100 mg wet-weight tissue, and the glycolysis between 20 to 120 min was used to compute the hourly rate.

The retention of carbon dioxide by the added bases has not been allowed for, as its effect on the results is considered to be negligible.

Respiratory activity

This was also estimated in the Warburg apparatus, by measuring rates of oxygen uptake with glucose as substrate (gas phase: 100 % O₂). Filter paper + 0·2 ml. of 10 % KOH solution were added to the centre well. Sodium phosphate buffer was used (pH 7·5).

Glycolysis and respiration media

A simple 'basic' medium was used consisting of NaCl, 0·13 M; KCl, 0·006 M; and NaHCO₃, 0·028 M. (Na₂HPO₄, 0·013 M was substituted for NaHCO₃ and the NaCl increased to 0·145 M in respiration experiments). Glucose (0·02 M) was added unless otherwise stated. Calcium was added to the medium when necessary at the start of the experiment.

Estimation of DPN-ase

Guinea-pig brain was removed as described above and homogenized in a Ten Broeck grinder containing the following medium: NaCl, 0·13 M; KCl, 0·006 M; NaHCO₃, 9 × 10⁻³ M (10 ml./g of brain). The resulting suspension was lightly centrifuged, the supernatant discarded, and the precipitate resuspended in fresh medium to the original concentration. A solution of diphosphopyridine nucleotide (DPN-potassium salt 60 % purity) in medium (10 mg/ml.) was neutralized with NaHCO₃ and placed in the side arms of Warburg vessels (0·5 ml./vessel), giving a final concentration of approximately 1·5 × 10⁻³ M-DPN.
Brain homogenate, 0.3 ml., was placed in the centre compartment together with any inhibitor. Thus the enzyme was allowed to remain in contact with inhibitor, and in the absence of substrate, for approximately 20 min. After gassing with 93% N₂ + 7% CO₂ (pH 7.0) and equilibration, the DPN was tipped in, and a zero reading taken after 3 min. Readings were taken after 10 and 20 min, during which time a reasonably steady rate of CO₂ production was maintained. The decrease in rate of CO₂ evolution in the presence of an inhibitor during this 3 to 23 min period was taken as a measure of DPN-ase inhibition.

Organic substances investigated

(a) Basic organic compounds

Pyrrole and quinoline obtained commercially were redistilled before use. Aniline, acetamide, α-naphthylamine, nicotinamide, benzidine, pyridine, 2:4-lutidene, triethylamine, choline (chloride) and nicotine were obtained commercially in the highest available purity. P-chloraniline of commercial origin was recrystallized from ethanol. N-methyl pyridine (iodide) was prepared and kindly given to us by Dr J. H. Fellman of this Institute.

(b) Sodium salts of organic acids

Sodium acetate, benzoate, chloroacetate, salicylate, α-ketoglutarate and pyruvate were obtained commercially in reagent purity.

The dissociation constants (pKₐ) of the acids and bases were taken from the International Critical Tables or the original literature. These substances were used at 37.5°C during the work to be described, but dissociation constants are normally quoted for 25°C. Consequently 25°C values have been used, and K_w has been taken as 10⁻¹⁴ (25°C) in order to convert pKₐ values to pKₐ when necessary.

Values of pKₐ obtained from the literature are as follows:

- aniline 4.6
- pyrrole 0.4
- pyridine 5.4
- quinoline 4.9
- α-picoline 6.1
- p-chloraniline 3.3
- nicotinamide 3.4

Results

Effects of calcium on anaerobic glycolysis of brain slices

It was first shown that the rate of anaerobic glycolysis of brain slices is increased by the presence of calcium ions in the medium, in confirmation of the results of Quastel & Wheatley (1937a). Since these authors found that the removal of traces of O₂ from their N₂ + CO₂ had no effect on the calcium stimulation, the unpurified gas mixture was used in the present work. The results given in figure 1 show that the stimulation of glycolysis by calcium ions reached a maximum at a concentration...
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of 4 mM, and then fell slowly up to 10 mM, the highest concentration tested. The maximum rate of glycolysis was three to four times the rate in the absence of calcium. The rate of glucose disappearance from the medium was measured in the presence of optimal calcium and in its absence and was found to correspond approximately with the CO₂ evolved (table 1).

**Figure 1.** *a*, effect of increasing concentrations of calcium on the anaerobic glycolysis of guinea-pig brain slices. *b*, increase in anaerobic fructolysis resulting from the addition of calcium to the medium.

- , rate in the absence of calcium; O, rate in the presence of 4 mM calcium.

**Table 1. Correlation of Anaerobic CO₂ Evolution and Glucose Disappearance in the Presence and Absence of Calcium**

Guinea-pig brain slices (approximately 100 mg per vessel). pH 7.5.

<table>
<thead>
<tr>
<th></th>
<th>µl. CO₂ evolved in 2h</th>
<th>glucose disappearance (mg)</th>
<th>glucose equivalent to CO₂ evolved (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>calcium absent</td>
<td>124</td>
<td>0.54</td>
<td>0.49</td>
</tr>
<tr>
<td>calcium present (4 mM)</td>
<td>421</td>
<td>1.7</td>
<td>1.6</td>
</tr>
<tr>
<td>calcium present/calcium absent</td>
<td>3.4</td>
<td>3.6</td>
<td>—</td>
</tr>
</tbody>
</table>

Fructose glycolysis, although proceeding at a much slower rate, was also markedly increased in the presence of 4 mM calcium (figure 1).

As Lasnitzki (1933) reported that tumour anaerobic glycolysis was accelerated by calcium, the effect of calcium on glycolysis by sarcoma 37 and Novikoff hepatoma...
slices was investigated. The results given in table 2 show, however, that high activities were obtained in the absence of calcium and that little or no stimulation in the rate of glycolysis was observed with calcium concentrations up to 10 mM.

**Table 2. Effects of varying concentrations of calcium on the anaerobic glycolysis of tumour slices**

Glycolysis is expressed as μl. CO₂/100 mg wet wt./h.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Calcium concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Novikoff hepatoma</td>
<td>220</td>
</tr>
<tr>
<td>Novikoff hepatoma</td>
<td>279</td>
</tr>
<tr>
<td>Novikoff hepatoma</td>
<td>202</td>
</tr>
<tr>
<td>Novikoff hepatoma</td>
<td>417</td>
</tr>
<tr>
<td>Sarcoma 37</td>
<td>292</td>
</tr>
<tr>
<td>Sarcoma 37</td>
<td>254</td>
</tr>
<tr>
<td>Sarcoma 37</td>
<td>240</td>
</tr>
</tbody>
</table>

**Mechanism of calcium stimulation**

Experiments were then carried out to see if it was possible to increase the rate of glycolysis of brain slices, in the absence of calcium, to a point where the addition of calcium resulted in no stimulation of activity. The following results were obtained.

(a) Variation of the atmospheric conditions to which the brain slices were exposed

Brain slices were gassed with a mixture of 93% O₂ + 7% CO₂ for 20 min, and then with 93% N₂ + 7% CO₂. The glycolytic activities in the presence and absence of calcium were compared with those of slices gassed with 93% N₂ + 7% CO₂ only. As the results in table 3 show, the activity of the slices pretreated with O₂ + CO₂ was increased in both the presence and absence of calcium, but the effect of the pretreatment was much greater in the latter case. In further experiments brain slices were exposed for 15 min to N₂ + CO₂, followed by 15 min exposure to O₂ + CO₂, and finally exposed to N₂ + CO₂ for 8 min. The glycolytic activity of the pre-exposed slices, in the absence of calcium, was slightly higher than in the presence of calcium, and both were higher than the calcium stimulated rate in the slices exposed only to N₂ + CO₂. In all cases the rates of glycolysis remained quite steady for the duration of the experiment.

Elliott & Henry (1946) using brain suspensions found that the rates of anaerobic glycolysis could be altered by freeing their gas mixtures from traces of O₂. The main discrepancy was found in preparations which were pre-oxygenated before being gassed with N₂ + CO₂, and they suggested that a store of pyruvate built up by oxygenation is maintained after transfer to commercial N₂ + CO₂ by the O₂ impurity, but disappears slowly in an O₂-free gas mixture. A possible explanation, therefore, of the results given in table 3 is that a store of pyruvate is built up in
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The brain tissue during oxygenation, and that its effect is similar to that of added
Calcium. The experiment with gas transferences in the order

\[ N_2 + CO_2 \rightarrow O_2 + CO_2 \rightarrow N_2 + CO_2 \]

was repeated, using a \( N_2 + CO_2 \) mixture freed from traces of \( O_2 \). If pyruvate
reformation were the cause of the high activity in the absence of calcium, a fall-off
in rate should be observed after transferring the slices to pure \( N_2 + CO_2 \), due to
depletion of the pyruvate formed. The results are given in figure 2 and show that
the glycolytic rate began rapidly but then fell off sharply. The results in figure 2
also show that the use of purified \( N_2 + CO_2 \), with the slices not previously exposed

### Table 3. Anaerobic glycolysis rates in brain slices pretreated with

\( O_2 \) and \( CO_2 \) in the presence and absence of calcium at pH 7.5

Glycolysis is given as \( \mu l. \ CO_2/100 \ mg \ wet \ wt./h. \) The mixture of nitrogen and carbon
dioxide was not purified from traces of oxygen (see figure 2).

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Glycolysis rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calcium absent</td>
</tr>
<tr>
<td>93% ( O_2 + 7 % CO_2 ) for 20 min, then 93% ( N_2 + 7 % CO_2 )</td>
<td>96</td>
</tr>
<tr>
<td>Control 93% ( N_2 + 7 % CO_2 )</td>
<td>31</td>
</tr>
<tr>
<td>93% ( N_2 + 7 % CO_2 ) for 15 min, then 93% ( O_2 + 7 % CO_2 ) for 15 min, then 93% ( N_2 + 7 % CO_2 )</td>
<td>(a) (b)</td>
</tr>
<tr>
<td>Control 93% ( N_2 + 7 % CO_2 ) only</td>
<td>192</td>
</tr>
<tr>
<td>Control 93% ( N_2 + 7 % CO_2 ) only</td>
<td>45</td>
</tr>
</tbody>
</table>

**Figure 2.** Anaerobic glycolysis of brain tissue after pre-gassing with oxygen-free \( N_2 + CO_2 \),
changing to \( O_2 + CO_2 \) and returning to oxygen-free \( N_2 + CO_2 \) in the presence and absence
of calcium, compared with that of tissue treated with oxygen-free \( N_2 + CO_2 \) only. The
figure shows that in the absence of calcium the anaerobic glycolysis of pre-gassed tissue
began rapidly but fell off sharply.

- ○, \( N_2 + CO_2 \) only, calcium present;
- ●, \( N_2 + CO_2 \) only, calcium absent;
- □, pre-gassed, calcium present;
- ●, pre-gassed, calcium absent.
to O₂, gave glycolytic rates in the presence and absence of calcium quite similar to those obtained with unpurified N₂+CO₂, in agreement with results of Quastel & Wheatley (19376).

This experiment strongly suggested that a pyruvate effect was at least partly responsible for the high activity in pre-oxygenated slices in the absence of calcium.

It is worth noting that allowing brain slices to stand for 30 min at 25°C on the bench in the 'basic' medium had no effect on the subsequent anaerobic glycolytic rate at pH 7·5 in the absence of calcium, and slightly reduced the rate in its presence. When this experiment was repeated with glucose omitted from the medium, while the slices were standing, a decreased rate of glycolysis resulted in both the presence and absence of calcium. Subjecting the slices to pH 6 for 8 min at 0°C reduced both rates slightly, while exposure to pH 4 for the same time resulted in an almost complete loss of activity (table 4).

### Table 4. Anaerobic Glycolysis in Brain Slices after Treatments as Noted Below

Rates were determined in the presence and absence of calcium and are given as Q₁₈₄. This method of expressing glycolysis is adopted because slices were weighed into the vessels after treatment and considerable variations in water content were observed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium Absent</th>
<th>4 mm Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Standing for 30 min at 25°C:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Glucose Present</td>
<td>2·8</td>
<td>10·1</td>
</tr>
<tr>
<td>(b) Glucose Absent</td>
<td>2·0</td>
<td>6·0</td>
</tr>
<tr>
<td>Control</td>
<td>2·7</td>
<td>12·9</td>
</tr>
<tr>
<td>(B) Placing in medium at pH 6 for 8 min at 0°C:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Glucose Present</td>
<td>3·2</td>
<td>7·8</td>
</tr>
<tr>
<td>(b) Ditto at pH 4</td>
<td>1·0</td>
<td>1·0</td>
</tr>
<tr>
<td>Control</td>
<td>3·8</td>
<td>9·5</td>
</tr>
</tbody>
</table>

(b) Effects of change of pH

A study was then made of the effects of variation of pH on the glycolytic rate in the presence and absence of calcium. The pH of the medium was varied between 7·0 and 7·6 by altering the bicarbonate concentration, and appropriate changes in sodium chloride content were made to maintain [Na⁺] constant. Under these conditions the rate of glycolysis in the presence of calcium remained substantially unaltered. In the absence of calcium, however, the rate increased progressively with falling pH, and at pH 7·0 practically equalled that with calcium present. These results (figure 3) show that the glycolytic activity of brain slices is markedly lowered by absence of calcium only at a pH above 7·0.

This effect might possibly be due to a 'bicarbonate inhibition', since the concentration of bicarbonate increases progressively with pH. Accordingly, an experiment was carried out in a calcium-free medium, using 9×10⁻³ M bicarbonate and the gas mixtures 93% N₂+7% CO₂ (pH 7·0) and 97·5% N₂+2·5% CO₂ (pH 7·4), obtained by dilution with nitrogen. The activity at pH 7·0 was approximately
double that at pH 7.4, in satisfactory agreement with the results obtained with
nitrogen mixtures containing 7% CO₂ and the appropriate bicarbonate con-
centrations giving pH 7.0 and 7.4 (figure 3). The activities at pH 7.0 and 7.6 were
also examined, using N₂ + CO₂ mixtures freed from O₂. The results were the same
as those obtained with the unpurified gas (figure 3).

**Figure 3.**

- **a.** Effect of varying the pH of the medium (by altering the bicarbonate concen-
tration) on the anaerobic glycolysis of brain slices, in the presence and absence of calcium.
  - ○, pH 7.6, 4 mM calcium present;
  - ▲, pH 7.0, 4 mM calcium present;
  - ●, pH 7.6, no calcium present;
  - ■, pH 7.2, no calcium present;
  - △, pH 7.0, no calcium present.

- **b.** Effect of varying the pH of the medium from 7.4 to 7.0 by increasing CO₂ concentra-
tion (bicarbonate constant) on brain anaerobic glycolysis in the presence and absence of
calcium.
  - x, pH 7.4, 4 mM calcium present;
  - ▲, pH 7.0, 4 mM calcium present;
  - △, pH 7.0, no calcium present.

- **c.** Part of a repeated using oxygen-free N₂ + CO₂.
  - ○, pH 7.6, 4 mM calcium present;
  - ●, pH 7.6, no calcium present;
  - △, pH 7.0, no calcium present.

(c) The effect of added sodium pyruvate

The results already described with pre-oxygenated brain slices indicated that
the high glycolytic activity in the absence of calcium might be due to the formation
of pyruvate. Pyruvate has long been known as a glycolytic stimulant (Mendel,
Bauch & Strelitz 1931) but does not appear to have been investigated from the
point of view of simulating the action of calcium in brain slices. For the present work an unpurified $N_2 + CO_2$ mixture was used, since it is likely that loss of pyruvate from the system is thereby prevented (Elliott & Henry 1946). When pyruvate (1 mM) was added to the 'basic' medium containing an optimal concentration of calcium (4 mM), a slight (10 to 20%) increase in glycolytic rate was observed. However, a very marked stimulation was obtained in the absence of calcium (figure 4a). Using a calcium-free medium, a curve of activity against pyruvate concentration was constructed, showing that 1 mM (or less) produced an optimal effect (figure 4b).

![Figure 4. a, effect on brain slice anaerobic glycolysis of adding calcium, sodium pyruvate and calcium + pyruvate to the medium. 
- calcium and pyruvate absent; 
- calcium (4 mM) present; 
- calcium (4 mM) + pyruvate (1 mM)

b, effect of increasing concentrations of sodium pyruvate on the anaerobic glycolysis of brain slices in a calcium-free medium. The broken line shows the glycolysis rate in the presence of 4 mM calcium.](http://rspb.royalsocietypublishing.org/)

(d) Replacement of calcium by organic bases at pH 7.6

Quastel & Wheatley (1937a) stated that the organic bases pyridine, pyrrole and nicotine stimulated the anaerobic glycolysis of brain cortex slices. This effect was further investigated. In preliminary experiments with pyridine, using basic medium containing an optimal calcium concentration, little or no stimulation of glycolysis was obtained. However, pyridine was found to stimulate anaerobic glycolysis very markedly in a calcium-free medium. The results given in figure 5 show that the addition of 32 mM pyridine to a calcium-free medium approximately trebled the
The rate of glycolysis. Calcium in optimal (4 mM) concentration was more effective than pyridine, and increased the rate about four times, but no increase above the calcium-stimulated level was seen in the presence of both substances. It appears, therefore, that calcium may be largely replaced by pyridine in the glycolysis medium.

The experiment was repeated with an oxygen-free N₂ + CO₂ mixture, but the same results were obtained (figure 5). Further studies were therefore continued with the unpurified N₂ + CO₂ mixture.

**Figure 5.** a, effect on brain anaerobic glycolysis in a calcium-free medium of the addition of calcium, pyridine and calcium + pyridine. Gas mixture N₂ + CO₂ not purified from traces of oxygen.

- ○, calcium absent;
- □, pyridine (32 mM) present;
- ●, calcium (4 mM) present;
- ■, calcium + pyridine present.

b, details as for a but the gas mixture was freed from traces of oxygen before use.

Other organic bases were found to stimulate, to varying degrees, the anaerobic glycolysis of brain slices in a calcium-free medium. Curves were constructed relating glycolytic rate to concentration of added base, and the maximum rate compared in each experiment with that given by 4 mM calcium. Considerable variations were seen in the shapes of the curves, and in the concentrations of the bases giving optimal stimulation: pyrrole, 32 mM; p-chloraniline, 3.2 mM; benzidine, 1.6 mM; α-naphthylamine, 1.6 mM; 2:4-lutidine, 16 to 32 mM; pyridine, 32 mM; quinoline, 1.6 mM; aniline, 16 mM; triethylamine, 64 mM; choline and V, N-methylpyridine, 16 to 32 mM; urea, 64 mM. Pyrrole and pyridine, for example, had high (32 mM) optimum concentrations, but the curves were relatively flat, resembling calcium in this respect. Quinoline, on the other hand, had a low optimum concentration (1.6 mM), but the curve fell very steeply at higher concentrations (figure 6).
Figure 6. Effect of increasing concentrations of organic bases on the anaerobic glycolysis of brain slices in calcium-free media. a, pyridine; b, pyrrole; c, quinoline.

Figure 7. Optimal stimulation of brain slice anaerobic glycolysis in calcium-free media by organic bases, related to the dissociation constant ($pK_a$) of the base. The broken line shows the stimulation given by 4 mM calcium. 1, Acetamide; 2, urea; 3, pyrrole; 4, p-chloraniline; 5, a-naphthylamine; 6, benzidine; 7, quinoline; 8, aniline; 9, pyridine; 10, 2:4-lutidine; 11, triethylamine; 12, N-methyl pyridine (iodide); 13, choline (chloride).
The stimulation of glycolysis effected by each substance was related to the calcium stimulation by the following formula, the term 'stimulation' being defined in this manner:

$$\text{Stimulation} = 100 \times \frac{\left( \frac{\text{CO}_2 \text{ evolution in the presence of an optimum concentration of added substance}}{\text{medium}} \right) - \left( \frac{\text{CO}_2 \text{ evolution in calcium-free medium}}{\text{medium}} \right)}{\left( \frac{\text{CO}_2 \text{ evolution in the presence of } 4 \text{ mM calcium}}{\text{medium}} \right) - \left( \frac{\text{CO}_2 \text{ evolution in calcium-free medium}}{\text{medium}} \right)}$$

**Relation of optimal stimulation to basic strength of added substance**

It was then found that, as a general rule, the optimal stimulation obtained with a given substance could be related to its basic strength. The collected results are given in figure 7, which shows a curve of optimal stimulation against $pK_a$. The most active substances were weak bases with $pK_a$ values lying approximately between 0.4 and 4.0. The substances in this group (pyrrole, $p$-chloraniline, $\alpha$-naphthylamine and benzidine) were more efficient glycolysis stimulators than calcium ions. The salts of the strong bases, choline and $N$-methyl pyridine, were very weak stimulators.

**Exceptions to the preceding generalization**

Nicotinamide ($pK_a$ for the pyridine $N = 3.4$) (Jellinck & Wayne 1951) was also tested as a glycolytic stimulant in calcium-free media, in a similar manner to the bases just described. However, the optimal stimulation of 85% ($Ca^{2+} = 100$), although substantial, fell significantly short of that expected (see figure 7) for a substance having this $pK_a$. Nicotinamide was also exceptional in its very high optimal concentration (0.2m). Creatinine ($pK_a 3.6$) entirely failed to stimulate anaerobic glycolysis. These exceptions make it clear that other properties besides basic strength must also be taken into account.

**Effects of organic acids, other than pyruvic, on anaerobic glycolysis of brain**

The sodium salts of various organic acids ($pK_a$ values ranging from 2.9 to 4.8) were also tested in calcium-free media, after being brought to pH 7.5 with sodium bicarbonate (table 5). Acetate and benzoate had no measurable effect on anaerobic glycolysis, while chloracetate gave a definite inhibition. A slight stimulation was observed with salicylate. $\alpha$-Ketoglutarate was inactive. It seems therefore that the effect of pyruvate already described is a specific one and is probably connected with its effect on the $DPN/DPNH$ ratio through lactic dehydrogenase activity. The failure of $\alpha$-ketoglutarate to accelerate anaerobic glycolysis in calcium-free media supports this view.

**Effects of organic bases on anaerobic glycolysis of tumour tissues**

Pyridine had little effect on the anaerobic glycolysis of sarcoma 37 or Novikoff hepatoma slices (calcium absent). No increase in rate was observed. Pyrrole was
without effect on sarcoma 37 glycolysis. These results, given in table 6, may be correlated with the fact that calcium itself failed to influence anaerobic glycolysis in these tissues.

**Table 5. Effects of Organic Acids (Na salts) on the Anaerobic Glycolysis of Brain Slices in the Absence of Calcium**

For comparison, vessels containing optimum concentrations of calcium were included in each experiment. Glycolytic rates are expressed in $\mu l$ CO$_2$/100 mg wet wt./h. pH 7.5.

<table>
<thead>
<tr>
<th>Acid</th>
<th>$pK_a$</th>
<th>0</th>
<th>0.64</th>
<th>1.6</th>
<th>3.2</th>
<th>8.0</th>
<th>16.0</th>
<th>32.0</th>
<th>4 mm calcium (Ca = 100) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetic</td>
<td>4.8</td>
<td>38</td>
<td>37</td>
<td>39</td>
<td>32</td>
<td>33</td>
<td>32</td>
<td>—</td>
<td>234</td>
</tr>
<tr>
<td>benzoic</td>
<td>4.2</td>
<td>45</td>
<td>—</td>
<td>45</td>
<td>48</td>
<td>41</td>
<td>52</td>
<td>39</td>
<td>182</td>
</tr>
<tr>
<td>salicylic</td>
<td>3.0</td>
<td>46</td>
<td>51</td>
<td>60</td>
<td>65</td>
<td>75</td>
<td>60</td>
<td>—</td>
<td>153</td>
</tr>
<tr>
<td>chloracetic</td>
<td>2.9</td>
<td>76</td>
<td>46</td>
<td>49</td>
<td>21</td>
<td>26</td>
<td>16</td>
<td>—</td>
<td>152</td>
</tr>
<tr>
<td>$\alpha$-ketoglutaric</td>
<td>2.9</td>
<td>37</td>
<td>38</td>
<td>36</td>
<td>30</td>
<td>36</td>
<td>—</td>
<td>—</td>
<td>155</td>
</tr>
</tbody>
</table>

**Table 6. Effects of Varying Concentrations of Pyridine and Pyrrole on the Anaerobic Glycolysis of Tumour Slices**

Glycolysis is given as $\mu l$ CO$_2$/100 mg wet wt./h.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Pyridine concentration (mM)</th>
<th>6.4</th>
<th>16</th>
<th>32</th>
<th>64</th>
</tr>
</thead>
<tbody>
<tr>
<td>nil</td>
<td>252</td>
<td>252</td>
<td>242</td>
<td>222</td>
<td>176</td>
</tr>
<tr>
<td>sarcoma 37</td>
<td>417</td>
<td>386</td>
<td>370</td>
<td>420</td>
<td>415</td>
</tr>
<tr>
<td>Novikoff</td>
<td>202</td>
<td>—</td>
<td>176</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Hepatoma</td>
<td>240</td>
<td>230</td>
<td>243</td>
<td>227</td>
<td>227</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pyrrole concentration (mM)</th>
<th>1.6</th>
<th>3.2</th>
<th>6.4</th>
<th>16</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>nil</td>
<td>240</td>
<td>230</td>
<td>243</td>
<td>227</td>
<td>263</td>
</tr>
</tbody>
</table>

**Inhibition of DPN-ase**

It has already been stated that nicotinamide stimulates glycolysis actively in calcium-free media. Nicotinamide was shown to be an inhibitor of DPN-ase by Mann & Quastel (1941), and has since been used extensively in homogenates to protect the DPN present. It seemed possible, therefore, that the organic bases might stimulate anaerobic glycolysis by protecting DPN in the slices. McIlwain (1950) examined the effects of a variety of substances on DPN-ase activity. Whilst confirming the inhibitory effect of nicotinamide, he pointed out that various pyridine derivatives, including nicotine and pyridyl-glyoxaline, are also inhibitory. Calcium ions (0.02 m), however, were without effect.

The efficiency of a number of bases as DPN-ase inhibitors was tested directly, using guinea-pig brain homogenate as a source of DPN-ase and DPN as substrate. No correlation was found between the ability of an organic base to inhibit DPN-ase and its efficiency as an anaerobic glycolytic stimulant. Pyrrole, one of the most active stimulators, had no measurable inhibitory effect on DPN-ase. Aniline
inhibited slightly, and triethylamine not at all. Pyridine and quinoline, which have an effect on anaerobic glycolysis comparable with that of aniline, were found to be active DPN-ase inhibitors, and do not seem to have been previously reported as such. Pyridine in fact was appreciably more active than nicotinamide. These results are shown in figure 8.

Factors influencing the anaerobic glycolysis of brain and tumour

The potency of the pyridine derivatives as DPN-ase inhibitors seemed to bear an inverse relationship to their dissociation constants (pyridine 5.4 > quinoline 4.9 > nicotinamide 3.4). Two other compounds with dissociation constants greater than pyridine (β-picoline 6.1 and nicotine 7.8) were also tested as DPN-ase inhibitors. A plot of the reciprocal of the inhibitor concentrations giving 35% inhibition (I₃₅) against pKₐ (figure 9) shows that there is a sharp fall between pyridine and β-picoline. The results suggest that pKₐ is a factor concerned with the potency of these bases in the inhibition of DPN-ase.

Effects of organic bases on brain-slice respiration

The effects of quinoline and pyrrole on brain-slice respiration were investigated, and the inhibitions obtained were compared with that given by 4 mM calcium. The results (figure 10) show that 1.6 mM quinoline and 32 mM pyrrole affect respiration only to about the same extent as 4 mM calcium. However, 6.4 mM quinoline inhibited respiration completely, suggesting that this concentration might exert a 'narcotic' effect in physiological preparations.
Effects of mixtures of calcium, organic bases and pyruvate on anaerobic glycolysis of brain

The results in table 7 show that mixtures of calcium ions, an organic base and sodium pyruvate at their optimal stimulating concentrations gave rates of anaerobic glycolysis that were no greater than that of the most effective constituent of the mixture. The absence of additive effects points to a common site of action of all the stimulating substances or to a common effect on a rate-limiting step.

\[ \text{Figure 9. Relationship between } DPN\text{-ase inhibition and } pK_a \text{ for pyridine derivatives. The molar concentration of each substance giving 35% inhibition (I}_{35}\text{) has been taken as a measure of potency, and the reciprocal of this concentration (1/I}_{35}\text{) plotted against pK}_a. 1, nicotinamide; 2, quinoline; 3, pyridine; 4, } \alpha\text{-picoline; 5, nicotine.} \]

Table 7. Anaerobic glycolysis of guinea-pig brain slices in the presence of combinations of calcium ions, pyridine and sodium pyruvate at their optimal stimulating concentrations

<table>
<thead>
<tr>
<th>additions to glucose medium</th>
<th>glycolysis (μl. CO₂/100 mg wet wt./h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>60, 58</td>
</tr>
<tr>
<td>4 mm calcium</td>
<td>195, 186</td>
</tr>
<tr>
<td>32 mm pyridine</td>
<td>160, 149</td>
</tr>
<tr>
<td>1 mm sodium pyruvate</td>
<td>235, 195</td>
</tr>
<tr>
<td>4 mm calcium + 32 mm pyridine</td>
<td>202, 186</td>
</tr>
<tr>
<td>32 mm pyridine + 1 mm sodium pyruvate</td>
<td>231, 197</td>
</tr>
<tr>
<td>4 mm calcium + 32 mm pyridine + 1 mm sodium pyruvate</td>
<td>242, 202</td>
</tr>
</tbody>
</table>

Physiological experiments with organic bases

Since these organic bases simulate the action of calcium in anaerobic glycolysis, it was thought of interest to investigate the possibility that they might replace calcium ions in neurophysiological systems. In co-operation with Professor F. C.
MacIntosh, F.R.S., and Mr R. I. Birks, of the Department of Physiology, McGill University, experiments were carried out on the perfused superior cervical ganglion of the cat. In this structure, as Harvey & MacIntosh (1940) have shown, the removal of Ca\textsuperscript{2+} from the perfusion fluid causes long-lasting spontaneous firing of ganglion cells, and failure of synaptic transmission owing to the inhibition of acetylcholine release from the preganglionic endings; both phenomena are rapidly reversed by the addition of Ca\textsuperscript{2+} to the fluid. The bases tested on the ganglion were

![Graph](http://rspb.royalsocietypublishing.org/)
quinoline, pyrrole and p-chloraniline. Each of these when added to the perfusion stream did, in fact, abolish the spontaneous firing of ganglion cells deprived of Ca\(^{2+}\); the effect was somewhat slower than when Ca\(^{2+}\) was added. The cessation of firing was, however, unaccompanied by restoration of synaptic transmission; moreover, both synaptic transmission and the response of the ganglion cells to exogenous acetylcholine were abolished by the bases even when Ca\(^{2+}\) was present. These effects on the ganglion were, in each case, produced at concentrations similar to those that gave optimal stimulation of anaerobic glycolysis in brain slices, and the relative potency of the three bases examined was similar to that observed in brain; quinoline and p-chloraniline were both about 10 to 15 times more effective than pyrrole. The inhibiting effects, which were completely reversible, were such as might be produced by a narcotic or a local anaesthetic. The action of these bases on excitable tissues generally could not, however, be described in such terms. Mr D. M. J. Quastel, of the Department of Physiology, McGill University, has tested the three bases mentioned above on the isolated rat-diaphragm preparation (Bulbring 1946), and has observed that each of them in threshold concentration potentiates the twitch response of the muscle to maximal stimulation via the phrenic nerve; at higher concentration this response is followed by depression or suppression of the twitch, and on washing out the drug a further long-sustained potentiation occurs. The relative potency of the three bases was again about the same as that seen with brain. The response of the muscle was in each case depressed or blocked when the base was present at about the lowest concentration that gave maximal stimulation of anaerobic glycolysis in brain, and potentiated when the concentration was 5 to 50 % of this. The mechanism by which the bases potentiate the twitch response has not been analyzed, but the effect shows that they are not acting merely as non-specific depressants of excitability. Narcotics and local anaesthetics do not act in this way; nor does calcium. Whether any of these physiological effects of the bases are exerted on cell sites specifically sensitive to calcium is unknown. It appears significant, however, that the bases act on physiological systems at the same order of concentration, and with about the same relative potency, as has been found for their action on anaerobic glycolysis.

**DISCUSSION**

The results obtained in the present work on the influence of calcium ions and of organic bases on anaerobic glycolysis of intact brain and tumour tissue may be summarized as follows:

1. The inhibitory effect on the rate of anaerobic brain glycolysis, established by absence of calcium ions from the medium in which the brain slices are suspended, is largely overcome by decreasing the pH to 7.0.

2. The same effect is obtained by subjecting brain slices to oxygenation after a period of anaerobic treatment. This result can be explained on the basis of pyruvate formation during the period of oxygenation.

3. The presence of 1 mM sodium pyruvate accelerates anaerobic brain glycolysis very markedly in a calcium-free medium, but only slightly in the presence of optimal calcium.
4. A number of organic bases accelerate anaerobic brain glycolysis in a calcium-free medium. Their efficiency in replacing calcium ions can be in general correlated with their dissociation constants.

5. The effects of the organic bases cannot be explained in terms of DPN-ase inhibition.

6. Anaerobic glycolysis of tumour slices exhibits little or no calcium dependence, and is not affected by the organic bases that are effective with brain. No additive stimulant effects were observed in media containing combinations of calcium ions, an organic base and pyruvate. The glycolytic rate attained in these circumstances approximated to the rate given by the most active substance alone, i.e. with the substances tried there appeared to be no inhibition of a more active by a less active substance. No correlation could be found between the ability of a base to stimulate glycolysis and its ability to inhibit DPN-ase.

The fact that mixtures of the bases with calcium gave no additive stimulatory effects on anaerobic glycolysis suggests a common site of action. This may also be considered in connexion with the observation that pyruvate stimulated markedly in calcium-free media, but only slightly in the presence of optimum calcium, i.e. there was no additive effect when both substances were present. Apparently, therefore, all three types of substance must have a common site of action, or affect the same rate-limiting reactions.

Most interpretations of the effect of inorganic salts on the metabolism of glucose by brain indicate that the effects are due to variations in ion balance. It is possible that the effects are associated with changes in membrane permeability brought about by the ions, as suggested by Dickens & Greville (1935). Added pyruvate presumably influences glycolysis by helping to maintain DPN in the oxidized form and consequently stimulating the activity of triose phosphate dehydrogenase.

It seems certain that some special attribute of brain tissue is responsible for the observed enhancements in rate of anaerobic glucose breakdown due to calcium ions and various organic bases and that the cell membrane is implicated. Apart from the early statement of Dickens & Greville that in aerobic glycolysis only brain tissue is influenced by high concentrations of potassium ions, there is recent evidence, from brain homogenate experiments (e.g. Utter 1950), that much higher activities are obtained anaerobically by using potassium media. This activating action of potassium in brain homogenates is in complete contrast to its inhibitory effect on anaerobic glycolysis in brain slices. Recently Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari (1955) found a very low anaerobic glycolysis in brain slices incubated in a high potassium medium. No essential biochemical difference has been reported between the glycolytic systems of brain slices and of brain homogenates; yet the kinetics of the two systems are affected in different ways by potassium and calcium ions. A specific membrane effect seems, therefore, to be involved—specific because the effects reported with brain slices are not obtained with tumour slices. We have shown, in the present work, that the anaerobic glycolysis of tumour slices is affected very slightly, if at all, by the presence of calcium ions. It has also been shown that the effects of calcium ions on anaerobic glycolysis of intact brain may be given equally well by organic bases. This fact, as
well as the apparent relationship between stimulation and $pK_a$ among the organic bases themselves, suggests a physicochemical basis for their activity. The most likely property in common between the organic bases and calcium is their ability to form cations in solution. However, the ionic concentrations of the most active bases are extremely small at pH 7.5.

The results of the physiological tests also suggest that the bases are able to simulate the effect of calcium ions in preventing the spontaneous firing of cat cervical sympathetic ganglia, but there was no restoration of synaptic transmission. It was significant that the bases acted in physiological systems at the same concentrations, and with about the same relative potency, as was found for their activities in anaerobic glycolysis. New membrane equilibria seem to be established in the presence of the bases, reflected on the one hand by changed rates of glucose breakdown in the neurone and on the other by changed neurophysiological activities.

The replacement of calcium, in its effects on anaerobic brain glycolysis, by traces of pyruvate obviously cannot be explained in terms of chemical or physico-chemical similarities between the two substances. It is possible that calcium ions act by becoming part of the structural pattern of the cell membrane in such a way that the active transport of glucose into the neurone is facilitated. If this is so, it does not seem unreasonable to suppose that a similar change in cell-membrane equilibria may be brought about by a change in energy relations at the neurone surface. The known effect of pyruvate on the $DPN/DPNH$ system could be correlated with such a change if this system were actually associated with the cell membrane. In this connexion it is of interest to note that in the red blood cell $DPN\text{-}ase$ is located at the cell surface (Alivisatos & Denstedt 1951).

We suggest, therefore, that the neurone surface is an important rate-limiting factor in cellular glucose metabolism under anaerobic conditions. Changes in equilibria, at the neurone surface, leading to increased anaerobic glycolysis rates, may be secured in two ways, either by varying the external ionic balance or by increasing the surface $DPN/DPNH$ ratio. As both ways have the same ultimate effect, no additive effects on glycolytic rates will be found by combinations of the various types of substances having stimulating effects on anaerobic glycolysis.

We wish to thank most sincerely Professor F. C. MacIntosh, F.R.S., and the Department of Physiology, McGill University, for their interest in this work and for their ready co-operation in investigating the neurophysiological behaviour of the organic bases discussed in this paper. We also wish to acknowledge with gratitude financial aid from the National Cancer Institute of Canada towards that aspect of our work concerned with tumour enzymology.

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Factors influencing the anaerobic glycolysis of brain and tumour

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Serially balanced sequences in bioassay

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Some bioassay techniques involve applying a long sequence of doses on one or more subjects and measuring the response that occurs each time. In order to prevent trends in the responsiveness of a subject from inflating the experimental error, randomized block and Latin square designs have been adapted to these experiments. Such constraints take no account of any residual effect of a dose tending to increase or diminish the response associated with the subsequent dose.

A serially balanced sequence for any specified number of different doses has a constraint like that of randomized blocks and an additional property that each dose is balanced in respect of the doses that immediately precede it in its several repetitions. A number of properties of these sequences have been investigated and certain simple categories have been enumerated. Sequences for four different doses are likely to be the most useful for parallel line bioassays. The statistical analysis of such assays, under a simple model for residual effects, is discussed, with particular reference to the choice of validity tests and of potency estimators. Tables have been prepared that enable the tests and estimates to be formed immediately from linear functions of the responses.

Subsequent sections are concerned with the extension of these methods to assays involving a greater number of different doses, limitations on the utility of the sequences, and applications to other types of experiment. The paper ends with brief notes on the selection of a randomized sequence.

1. Introduction

Some bioassays can be conducted entirely on one subject. Typical is the histamine assay technique described by Schild (1942), in which a strip of guinea-pig's gut is exposed to successive doses of the standard and test preparation. Each dose can