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# THE STUDY AND ASSAY OF SUBSTANCES AFFECTING INTESTINAL ABSORPTION IN THE MOUSE

BY

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Knowledge of how substances are absorbed by the intestine is scanty (Davson, 1959). The discovery of the inhibitory effect of the cation hexadecyltrimethylammonium (cetrimonium) on intestinal absorption provides a new tool for further investigations (Nissim, 1960a,b,c). Precise and rapid methods of assay are needed in order to study the different problems of intestinal absorption as well as to test substances influencing this function. Of substances related to cetrimonium alone, a large number is available (Lawrence, 1950).

Methods hitherto used in the investigation of intestinal absorption include the intraluminal perfusion of the rat intestine either as an isolated preparation or in the anaesthetized animal. Both methods were improved by Smyth and co-workers (Jervis, Johnson, Sheff & Smyth, 1956; Smyth & Taylor, 1957). The intestinal perfusion method was used in the anaesthetized rat and rabbit to study the effect of cetrimonium on the absorption of three substances, namely glucose, sodium butyrate and methionine, in a single experiment (Nissim, 1960c). In the present work, the aim was to devise a method for the rapid assay of substances which inhibit or stimulate absorption, so that the percentage absorption of the nutrients studied had to be about 50% in control experiments. An absorption percentage is taken as the fraction of the nutrient absorbed out of the total originally used in the experiment, expressed as a percentage. It was therefore necessary as a first step to find the shortest time and the smallest quantities of fluid and nutrients which would give such a suitable absorption percentage.

The author has already shown that cetrimonium inhibits the absorption of glucose in the rat and rabbit, and of sodium butyrate and methionine in the rat (Nissim, 1960c). The inhibitory effect of phloridzin on glucose and on butyrate absorption is well known (Jervis *et al.*, 1956; Davson, 1959), but their precise relative activities have not been determined. These two substances were therefore selected for comparative study and assay by the new method.

## METHODS

The mice were pure C3H or CBA strain, 6 to 12 months old. The two types of perfusion apparatus used are described in the appendix. The control fluid consisted of saline (0.9% w/v) containing glucose, sodium butyrate and methionine in concentrations of 0.2% (w/v), these representing, respectively, carbohydrates, short-chain fatty acids and amino acids. Cetrimonium bromide and phloridzin (obtained from I.C.I. and Light & Co., respectively) were added to give concentrations of $10^{-5}$ to $10^{-2}$. All the solutions were approximately neutral.

TABLE 1

ABSORPTION OF GLUCOSE, SODIUM BUTYRATE, METHIONINE AND WATER IN CONTROL PERFUSION EXPERIMENTS

Absorption values are means and standard errors

| Expt. No. | Duration of perfusion (min) | Strain | Sex | No. of mice | Average weight of mice (g) | Length of small intestine (cm) | Absorption of: Glucose (mg/min) | Glucose (%) | Sodium butyrate (mg/min) | Sodium butyrate (%) | Methionine (mg/min) | Methionine (%) | Water (ml./hr) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 30 | C3H | ♂ | 15 | 26·5±0·7 | 30·4±0·5 | 0·57±0·02 | 34·2±1·2 | 0·61±0·03 | 36·4±1·7 | 0·77±0·04 | 46·4±2·2 | 2·35±0·21 |
| 2 | 30 | C3H | ♀ | 15 | 20·2±0·7 | 32·8±0·3 | 0·63±0·04 | 37·9±2·1 | 0·60±0·03 | 35·9±1·8 | 0·76±0·03 | 45·3±1·8 | 2·27±0·19 |
| | | | | | $P<0·001$ | $P<0·001$ | $P<0·01$ | | $P\simeq 0·9$ | | $P\simeq 0·8$ | | $P<0·01$ |
| 1 & 2 | 30 | C3H | ♂ & ♀ | 30 | 23·4±0·8 | 31·6±0·4 | 0·60±0·02 | 36·1±1·2 | 0·60±0·02 | 36·2±1·2 | 0·76±0·02 | 45·8±1·4 | 2·31±0·14 |
| 3 | 15 | C3H | ♀ | 15 | 19·7±1·7 | 34·4±0·7 | 0·75±0·03 | 56·3±2·0 | 0·66±0·02 | 49·8±1·4 | 0·70±0·03 | 52·5±1·8 | 2·57±0·16 |
| 4 | 5 | CBA | ♀ | 15 | 29·7±1·1 | 34·5±0·5 | 1·02±0·07 | 51·2±3·3 | 0·76±0·04 | 38·1±1·8 | 0·85±0·04 | 42·6±2·2 | 2·12±0·35 |

Control perfusion experiments were carried out on four groups of fifteen mice for 30, 30, 15 and 5 min respectively (Table 1). The animals, fasted of food and water overnight, were anaesthetized with 0.2 to 0.3 ml. of 0.6% (w/v) sodium pentobarbitone (Veterinary Nembutal, Abbott Laboratories) given subcutaneously. A further 0.05 ml. was given intraperitoneally if required, after the abdomen had been opened. The small intestine was washed with 6 ml. of 0.9% saline (37° C), and emptied by gentle manual compression from the duodenum to the terminal ileum. The inflow and outflow polyethylene tubes were tied in position at the pyloric and caecal end respectively. The perfusion pressure was adjusted at 10 cm of water, and this gave a flow rate of 4 to 6 ml./min. Care was taken that neither air bubbles nor plugs of mucus impeded this flow. A total quantity of 25, 10 or 5 ml. perfusion fluid was used in experiments lasting 30, 15 and 5 min, the quantities of nutrient substances corresponding to these volumes of fluid were 50, 20 and 10 mg, and the number of times the fluid circulated in these experiments was 6, 7.5 and 5 respectively. Time was measured with a stop-clock.

At the end of each experiment, the small intestine was clamped at both ends, and the fluid from apparatus and intestine was drained into a cylinder. Residual fluid in the reservoir and tubing was sucked with a pipette and the total volume measured. The net amount of fluid absorbed from the lumen or secreted into it per hour was calculated, and the percentage absorptions of glucose, sodium butyrate and methionine were obtained by the methods described previously (Nissim, 1960c).

## RESULTS

Control experiments are summarized in Table 1. The effect of sex, studied in two groups of fifteen C3H mice, is shown in the first two rows. Though members of the two sexes were litter-mates, the females were much smaller. Statistically significant differences between the sexes were found in the length of intestine and in the absorption of glucose and water, but these differences were small. Since the number of animals available from any single strain was limited, 30- and 15-min experiments were carried out on C3H mice and 5-min experiments on CBA mice.

The results of the assay with cetrimonium and phloridzin are given in Table 2 and Fig. 1. The effects of these drugs on glucose absorption is evident and there is good regression of response on logarithm of concentration. Cetrimonium showed an inhibitory effect on all three substances, but phloridzin on the absorption of glucose and butyrate only. The effect of cetrimonium on butyrate and methionine absorption was small.

TABLE 2

EFFECT OF CETRIMONIUM AND OF PHLORIDZIN ON THE ABSORPTION OF GLUCOSE, SODIUM BUTYRATE AND METHIONINE

Figures represent mean percentage absorptions and standard errors of three experiments. Concentrations are w/v in perfusion fluid

| Duration of experiment (min) | Concentration of drug (%) | Cetrimonium on absorption of | | | Phloridzin on absorption of | | |
|---|---|---|---|---|---|---|---|
| | | Glucose (%) | Sodium butyrate (%) | Methionine (%) | Glucose (%) | Sodium butyrate (%) | Methionine (%) |
| 30 | 0·001 | 45·5±3·3 | 32·9±2·8 | 36·7±2·2 | 29·0±5·2 | 28·4±4·0 | 46·1±5·2 |
| | 0·01 | 22·6±7·0 | 28·0±4·9 | 32·6±5·6 | 6·6±2·5 | 37·1±3·1 | 41·9±5·1 |
| | 0·1 | 3·4±2·0 | 26·0±1·4 | 34·2±1·5 | −3·6±0·6 | 13·9±6·2 | 48·8±3·8 |
| 5 | 0·001 | — | — | — | 38·8±2·6 | 42·6±2·2 | 40·8±2·8 |
| | 0·01 | 48·5±3·1 | 33·8±2·1 | 49·8±2·1 | 22·4±3·8 | 47·6±2·5 | 48·6±5·8 |
| | 0·1 | 22·7±1·8 | 30·1±1·5 | 37·1±3·0 | −3·4±2·5 | 32·2±1·7 | 42·5±4·6 |
| | 1·0 | 1·4±5·8 | 18·8±2·1 | 41·2±5·2 | — | — | — |

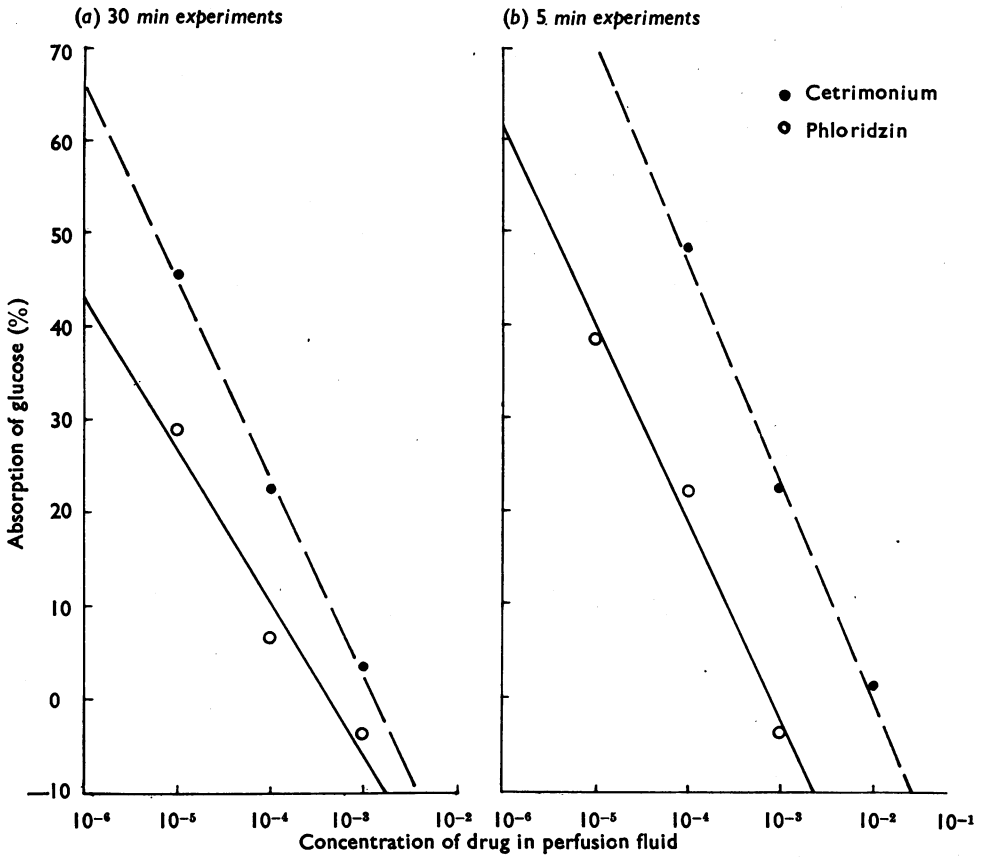


Fig. 1 (*a*). Log dose/response lines representing assay of the inhibitory activities of cetrimonium and phloridzin on the intestinal absorption of glucose by the 30-min intraluminal perfusion method in C3H male mice. Each point represents the mean of three experiments.
(*b*) Log dose/response lines representing assay of the inhibitory activities of cetrimonium and phloridzin on the intestinal absorption of glucose by the 5-min intraluminal perfusion method in CBA female mice. Each point represents the mean of three experiments.

TABLE 3

DOSE/RESPONSE RELATIONSHIPS, DIFFERENCES BETWEEN SLOPES, POTENCY RATIOS, AND ANALYSES OF VARIANCE FOR CETRIMONIUM AND PHLORIDZIN IN 30-MIN AND IN 5-MIN ASSAYS OF INHIBITORY EFFECT ON GLUCOSE ABSORPTION

y=response, x=log of dose

| | 30-Min experiments | 5-Min experiments |
|---|---|---|
| Dose/response equation for cetrimonium | y=66·03−21·05x | y=94·76−23·52x |
| Dose/response equation for phloridzin | y=43·22−16·28x | y=61·50−21·12x |
| Potency ratio of phloridzin/cetrimonium | 5·10 | 16·65 |
| Range of potency ratio at $P=0·05$ | 14·06−2·241 | 32·86−8·71 |
| *Analysis of variance* | | |
| Source of variation | | |
| Between phloridzin and cetrimonium | $P \simeq 0·001$ | $P \simeq 0·05$ |
| Linear regression | $P \ll 0·001$ | $P \ll 0·001$ |
| Deviation from regression | $P > 0·2$ | $P > 0·2$ |
| Departure from parallelism | $P > 0·2$ | $P > 0·2$ |
| Difference between slopes | $P > 0·2$ | $P > 0·5$ |

The effect of cetrimonium on the absorption of glucose, butyrate and methionine in 5-min experiments was similar to that in 30-min experiments but much weaker, so that the range of concentrations had to be increased tenfold. The effect of phloridzin in 5-min experiments was only slightly weaker.

The analysis of the assay for the effect of cetrimonium and phloridzin on glucose absorption is shown in Table 3, which includes a skeleton analysis of variance. In 30-min, as well as in 5-min experiments, the dependence of percentage absorption on the logarithm of the concentration is linear in the range of the concentrations used for both cetrimonium and phloridzin. Deviations from regression are not significant ($P>0.2$) and the slopes of the dose/response lines are similar.

## DISCUSSION

The results show that intestinal perfusion in the mouse offers a rapid, efficient and economical method for studying intestinal absorption as well as for screening compounds which promote or diminish absorption. The assays were deliberately limited to three animals per dose to demonstrate the efficiency of the method, since highly significant results have been obtained with small numbers. This efficiency is particularly evident in the case of glucose, for it is on the absorption of this substance that both cetrimonium and phloridzin exhibit their greatest effect. A much smaller inhibitory effect was obtained on the absorption of butyrate with both drugs, and on the absorption of methionine with cetrimonium. The relative ineffectiveness of these drugs in blocking butyrate and methionine absorption may be due to weak intrinsic activities or efficacies in the terminology of Ariëns & Groot (1954) and Stephenson (1956) respectively. Alternatively, the drugs may block only one of two or more pathways of butyrate and methionine absorption, so that only partial inhibition can be expected even when the full effect is exerted on its site of action.

Although female C3H mice are much smaller than their male litter-mates, their intestines are larger and longer. Glucose absorption is also greater, though net water absorption is less.

The total time spent over a 5-min perfusion experiment is 20 min, and over a 30-min experiment, 45 min. The 5-min experiments would probably be best for rapid screening of potentially effective compounds, while the 30-min experiments are best for precise assays of potency.

The absorption of nutrients declines with time, and greatest absorption seems to occur in the first 5 min (Table 1).

In 30-min experiments phloridzin is about five-times as active as cetrimonium; in 5-min experiments it is about seventeen-times as active (Table 3). Thus, cetrimonium reaches its full effect slowly and phloridzin quickly. The difference may be related to the ionic character of cetrimonium.

Since cetrimonium blocks the absorption of glucose by the intestinal mucosal cell in mice, rats and rabbits, it may also inhibit the absorption of glucose by bacterial cells, and this effect may be responsible for the germicidal activity of cetrimonium and related compounds.

## APPENDIX

### PERFUSION APPARATUS FOR RAPID INTESTINAL ABSORPTION ASSAYS IN SMALL ANIMALS*

BY

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The intestinal perfusion apparatus described here has been primarily designed for the rapid *in vivo* assay in the mouse of substances capable of influencing the intestinal absorption of nutrients, electrolytes, water or drugs. It can, however, be used for larger animals such as the rat or guinea-pig.

In principle, the apparatus is similar to that designed by Sheff & Smyth (1955) for the study of *in vivo* intestinal absorption in the rat, and used in previous experiments by one of us (Nissim, 1960c). The fluid is circulated by a stream of moist air or oxygen in both cases. However, the amount of fluid used in rat or rabbit experiments (50 to 250 ml.) was adequate to fill the perfused intestine and the dead space of the apparatus, and at the same time to ensure good circulation of the perfused fluid. In order to obtain a rapid as well as sensitive method for the study of intestinal absorption in a small animal such as the mouse, it was necessary to reduce the perfusion time and the amount of fluid without reducing the concentrations in the perfusion fluid of the nutrient substances under study. If $a$ denotes the minimum capacity of the apparatus or " dead space " and $b$ the maximum expected capacity of the mouse small intestine, then $a+b$ would represent the minimum amount of fluid which must be used in the experiment. Since a perfusion time of 5 min was considered desirable, at least in some of the assays, a flow rate of $a+b$ ml./min would evidently be required if the total fluid were to circulate five times during the perfusion experiment. The problem therefore resolved itself into finding the approximate volume of the minimum dead space $a$ to give a flow of $a+b$ ml./min. The value of $b$ was found to be 3.25 ml. in over 200 perfusion experiments. Although $a$ could be reduced to very small values by the use of narrow polyethylene tubing, a flow rate of $a+b$ ml./min was not secured except when certain mechanical conditions were fulfilled. These were determined largely by trial and error with the aid of a flowmeter incorporated in the circuit.

### DESIGN AND METHODS

Two types of apparatus were used in these experiments and are shown in Figs. 2 and 3; they are basically similar. In type 1 (Fig. 2), which is made of Perspex and has a capacity of 25 ml., the warming water-jacket is restricted to the perfusion reservoir itself, while in type 2 (Fig. 3), which is made of stainless steel and has a capacity of 75 ml., the water-jacket encloses in addition 10 cm lengths of inlet and outlet tubing. Type 1 would probably prove adequate for most if not all assays, and is certainly both simpler and cheaper to make. Stainless steel was used in type 2 to minimize risks of drug adsorption by the material of the apparatus, particularly when active compounds were studied.

Fig. 2, which is a diagrammatical representation of the first model, is self-explanatory and gives the essential features of the two forms of the apparatus. The fluid from the warmed (37° C) perfusion reservoir flows into the small intestine of the mouse through a narrow polyethylene tube (1.37-mm bore). On its

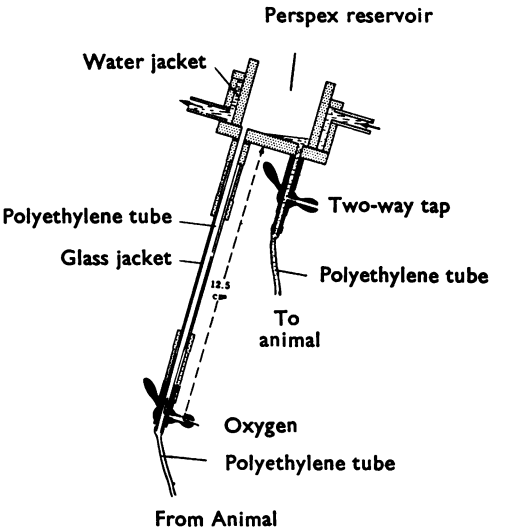



Fig. 2. Diagram of type 1 intestinal perfusion apparatus, with measurements of essential sections, to give total perfusion capacity of 5 ml. and a flow rate of 5 ml./min for the assay, in the mouse, of substances affecting intestinal absorption (scale, 10 : 3 reduction).

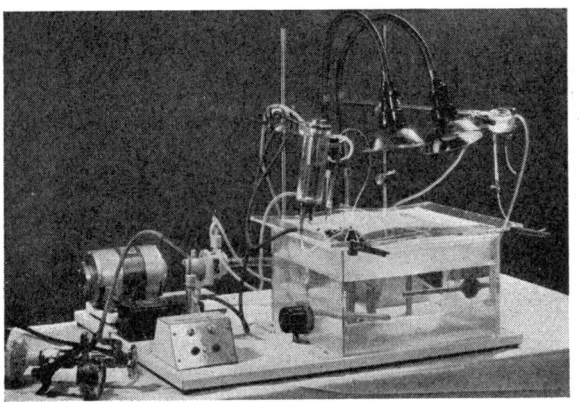
return from the animal, the fluid is lifted back to the reservoir by an adjustable stream of oxygen, which enters the system from a cylinder through the side-nozzle of a two-way tap. The section connecting the tap, through which the oxygen enters, to the perfusion reservoir consists of a polyethylene tube (2.75-mm bore) in a rigid glass tube to prevent bending. While the polyethylene tubing in the rest of the system and the bore of the two-way taps can have very narrow diameters, the section through which the oxygen rises and lifts the fluid into the reservoir against gravity has the optimum dimensions of $12.5 \times 0.275$ cm, as shown in the diagram. These dimensions are critical, for shorter length and narrower bore give smaller flow rates, while a wider bore gives greater dead space. The capacity of this section is therefore about 0.75 ml. A further 1·0 ml. of fluid is required to fill a small flowmeter (Figs. 3*a* and *b*) and the connecting tubes in the circuit and to ensure continuity of flow in the perfusion reservoir. The two components together give a value of 1.75 ml. for *a*.

The chromium-plated two-way taps shown in the diagram may be used for washing through the apparatus and intestine when a second test or assay is required on the same animal. A frame of stainless steel meshwork placed in the perfusion reservoir traps mucus and blood clots when larger animals are used, but seems unnecessary for mice.

The perfusion reservoir may be kept tilted at an angle of about 10° to assist the flow of fluid from inlet to outlet. The complete apparatus shown in Fig. 3*a* includes two units so that two experiments may be carried out simultaneously by two workers, or, in 30-min experiments, a second perfusion may be set up by the same worker while the first is still running. Electrical lamps are fitted to help in keeping the animal and apparatus warm during the experiment and to warm the tail of the animal for the collection of blood samples. Water is warmed in the large Perspex reservoir (under the animal in the photograph) to about 42° C and circulated through the two water-jackets by a single pump driven by a silent motor (Batwin Electric Motors, Richmond, Surrey). The temperature is under thermostatic control ($\pm 1$° C).

The perfusion pressure can be adjusted by altering the level of the perfusion reservoirs on their graduated stands. A manometer type of flowmeter with tantalum float (Fischer & Porter, Workington, Cumberland) can be used to check the rate of flow between experiments, or, if its capacity is sufficiently small, may be incorporated into the circuit as shown in Fig. 3*b*.

(*a*)



(*b*)

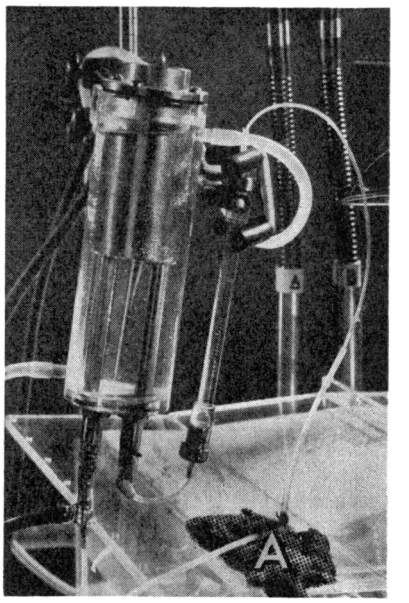


Fig. 3 (*a*). Photograph of a double intestinal perfusion unit for the rapid assay of substances affecting intestinal absorption in the mouse (A). On the right is shown type 1 Perspex perfusion apparatus. On the left is shown type 2 stainless steel perfusion apparatus complete with flowmeter and mouse in circuit. Note: (1) motor and pump to circulate water from big reservoir (thermostatically heated) to jacket of perfusion reservoir; (2) oxygen cylinder from which oxygen drives perfusion fluid through intestine of mouse; (3) infrared lamps for keeping mice warm during the experiment.
(*b*) Photograph of type 2 stainless steel perfusion apparatus of Fig. 3*a* with flowmeter in circuit.

## RESULTS AND DISCUSSION

Five ml. of fluid were used regularly in this perfusion apparatus and found adequate in 5-min assays. Type 2 has been used for perfusion volumes of 5 to 50 ml., but the perfusion reservoir can be enlarged to hold 100 ml. without changing the basic design.

The perfusion reservoir with tubing can be emptied of fluid efficiently at the end of an experiment by suction with a pipette. The error in fluid recovery does not usually exceed 0.1 ml. In 5-ml. perfusion experiments this represents an error of only 2%.

It seems impractical to use less than 5 ml. of fluid for perfusion in routine assays, not only because the mouse intestine itself may hold more than half this volume, but also because about 5 ml. of fluid would in most instances be needed for carrying out the chemical estimations at the end of the experiment. For simultaneous studies on the absorption of glucose, sodium butyrate and methionine, for instance, these estimations require 0.2, 1 and 2 ml. respectively, and the remaining 1.5 to 1.8 ml. may be needed for a check on a doubtful estimation.

## SUMMARY

1. A method of studying intestinal absorption in the anaesthetized mouse is described. Assays of the effect of cetrimonium and phloridzin on the absorption of glucose, sodium butyrate and methionine were performed.

2. The relative potencies of cetrimonium and phloridzin in reducing glucose absorption in 30-min and in 5-min perfusion experiments were 1 : 5 and 1 : 17 respectively. The greatly reduced activity of cetrimonium in 5-min experiments indicates that it reaches its site of action slowly, probably because of ionic charge.

3. The essential requirements of an apparatus for the rapid assay in the mouse of substances enhancing or inhibiting absorption are described in an appendix. Five ml. of fluid can be perfused intraluminally, of which only 1.75 ml. occupies the dead space of the apparatus and 3.25 ml. the mouse intestine. The minimum perfusion time is 5 min and the flow rate is 5 ml./min.

## NOTE ADDED IN PROOF

In all recent 30-min assays, four experiments (using type 2 apparatus) were mounted successively during the 30 min on the same perfusion unit, and taken down in the same order.

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