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# Effects of Insulin, Sodium and D-Glucose on Amino Acid Absorption in the Intestine of Rats

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APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

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Richard Tocher

Amino acid absorption across rat intestine in vitro was inhibited or stimulated by insulin depending on the hormone serosal concentration.

The absence of sodium ions from the incubating solution resulted in a significant decrease of L-alanine absorption. However, L-alanine absorption was enhanced in a sodium-free medium by the addition of insulin on the serosal side of the intestine.

A sizable decrease in L-alanine and L-lysine absorption was produced by introduction of D-glucose ( 0.2 % and 2 % ) in the Krebs-Ringer's incubating buffer. Nevertheless, the presence of D-glucose did not prevent the stimulatory effect of insulin on amino acid absorption.

# EFFECTS OF INSULIN, SODIUM AND D-GLUCOSE ON AMINO ACID ABSORPTION IN THE INTESTINE OF RATS

by

ANDRE-GERARD CRAAN

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TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

# INTRODUCTION

Amino Acid Absorption in the Small Intestine. Stomach enzymes attack only a small amount of the protein of the diet. Thus, a mixture of undigested protein with products of peptic digestion ranging from large polypeptides to a few free amino acids is delivered to the intestine as the protein portion of the diet. As a result, the major part of protein digestion must take place in the intestine (1).

Pancreatic enzymes (trypsin, chymotrypsin, carboxypeptidase) that are released into the intestinal lumen through pancreatic ducts hydrolyze some proteins to amino acids. However, most of the products of such hydrolyses are dipeptides and small polypeptides (2).

Enzymes (amino polypeptidases and dipeptidases) located in the epithelial cells of the small intestine break the undigested dipeptides and small polypeptides into amino acids (3) . The latter then pass into the blood stream.

Amino acid absorption is an active transport process supported by oxidative metabolism in the mucosa (2,4). As an active transport process, amino acid absorption may overcome the sum of the forces of diffusion, electrical field and solvent drag (5), and thus may go against a concentration gradient (6,7).

Insulin and Amino Acid Absorption. The hormone insulin has been the subject of many speculations and numerous investigations. Since it was found to alleviate diabetes (8,9,10,11), medical physiologists and biochemists have oriented their research intersts, for the most part, toward its effects on blood sugar level. As a result, the literature is rich in studies of insulin effects on the intestinal absorption of carbohydrates and especially of glu-

cose.

Compared to the vast literature concerning the effects of insulin and the effects of diabetes on the intestinal absorption of glucose, there has been very little work on the effects of insulin on intestinal amino acid absorption.

Insulin has been found to stimulate transfer of the non-metabolizable amino acid, α-aminoisobutyric acid, from extracellular to intracellular space in rat diaphragm (12,13). However, the results are different for natural metabolizable amino acids. Manchester and Young (13), and Wool and Krahl (14), indeed, found no effect of insulin on the accumulation of L-amino acids in the same tissue. Manchester and Krahl (15) found that insulin can promote amino acid incorporation into protein. A similar conclusion was reached for rat adipose tissue (16). This stimulatory action of insulin appears not to depend on its simultaneous enhancing action on amino acid transport (15,17).

The preceding investigations were centered more on amino acid incorporation into tissue protein than its actual transport into tissues. However, in a paper that dealt specifically with the effect of insulin on intestinal absorption Fromm et al. (18) denied any effect of insulin on L-alanine transfer by rabbit ileum in vitro.

Sodium and Amino Acid Absorption. Attention has been focused on sodium in absorption phenomena since it was discovered that membrane resting potential was changed as $Na^+$ moved in and out of myelinated fibers (19), the giant axon of the squid (20), and striated muscle fibers of frogs (21). These and other investigations (22,23,24) led to the idea of a sodium pump functional in membrane transport phenomena. The sodium pump is an energy-dependent system that transports sodium ions across cell membranes and

creates an electrochemical potential difference between intracellular and extracellular fluids.

It was not until the end of the 1950's that studies were made on the influence of $Na^+$ on the intestinal absorption of amino acids. It has been found that a decrease in $Na^+$ concentration in the medium causes a parallel decrease in amino acid transfer (25, 26, 27). However, Adibi's findings (28) do not support such a general conclusion.

Carbohydrates and Amino Acid Absorption. There appears to be no one unifying principle as to the role of carbohydrates on amino acid transfer across the intestine. Several workers (29, 30, 31, 32, 33) have found that glactose inhibits amino acid transport. Mannose is reported to have no effect (34), and reports are contradictory in regard to the effect of fructose on amino acid transport (33, 34).

Previous workers have disagreed on the role of glucose in the absorption of amino acids. In the words of Hardcastle et al. (29), "the reported effects of D-glucose vary from inhibiton (33, 35) to stimulation (32, 34, 36) or even no effect (37)." These investigations on D-glucose were all conducted using rat small intestine.

## MATERIALS

L-alanine-$C^{14}$(U), L-lysine-$C^{14}$(U) and α-aminoisobutyric-1-$C^{14}$ acid (New England Nuclear) were each diluted separately in 0.01 N HCl to make stock solutions with a final concentration of 5 microcuries per milliliter. Specific activities were 123 millicuries per millimole for L-alanine, 255 millicuries per millimole for L-lysine and 7.99 millicuries per millimole for α-aminoisobutyric acid.

To start each experiment, 20 to 60 microliters of the stock solution of one of the amino acids, corresponding to an activity of 0.1 to 0.3 microcurie, were transferred by microliter syringe to 7.5 milliliters of Krebs-Ringer's bicarbonate buffer which served as the incubating medium.

The composition of the Krebs-Ringer's bicarbonate buffer is as follows (38):

100 parts 0.90% NaCl

4 parts 1.15% KCl

3 parts 1.22% $CaCl_2$

1 part 2.11% $KH_2PO_4$

1 part 3.82% $MgSO_4 \cdot 7H_2O$

21 parts 1.30% $NaHCO_3$.

Sodium ions were replaced with potassium in some experiments. In these experiments, the bicarbonate buffer was made in the same proportion as shown in the table above, except that NaCl and $NaHCO_3$ were replaced by KCl and $KHCO_3$ on a percentage (w/v) basis. For the study of D-glucose effects on amino acid absorption, D-glucose was added to the incubating Krebs-Ringer's

bicarbonate solution at concentrations of 0.2% and 2% (w/v).

Fresh solutions of insulin with a concentration of 20 units per milliliter in 0.01 N HCl were prepared from crystalline bovine insulin (26 U/mg, CalBiochem) prior to each set of experiments. All solutions were stored in the cold and used within 3 days of preparation.

## METHODS

### The Everted-sac Technique

Sexually mature male Fischer/344 rats (Simonsen Laboratories, Gilroy, California), weighing 160-250 grams and ranging in age between 4 and 8 months, were kept on a 12 hours light-12 hours dark cycle. They were fasted 30-40 hours before they were sacrificed, after ether anesthesia, by a sharp blow on the head. The small intestine was dissected free and flushed thoroughly with saline unitl it became clean. The intestine was immediately turned inside out (39), placed in Ringer's solution and divided into 6 cm length sacs.

The sacs were identified as duodenal, jejunal and ileal sacs. They were incubated in a chilled Krebs-Ringer's solution that had been gassed with 95% $O_2$-5% $CO_2$. This procedure (Olsen and Rosenberg) was used in order to insure the viability of the intestinal tissue (40). The Olsen-Rosenberg technique was modified for about 10% of the experiments in which the intestinal sacs were left in normal saline exposed to the air at room temperature until they were used for absorption experiments. The period of maintenance in normal saline at room temperature varied from 2 to 40 minutes. No significant difference in amino acid absorption was noted between these tissues and the tissues that were given the Olsen-Rosenberg treatment.

Each everted experimental sac was filled with 0.5 to 5 units of insulin in 0.01 N HCl (20 Units/ml) made up to 0.75 cc with Krebs-Ringer's solution. An equal volume of 0.01 N HCl was added to the Krebs-Ringer's solution that was introduced into each control sac.

Individual sacs with a serosal volume of 0.75 cc were ligated at both ends, then incubated in 50 cc Erlenmeyer flasks containing 7.5cc of Krebs-Ringer's solution and $C^{14}$-amino acid. The flask was gassed with a mixture of 95% $O_2$-5% $CO_2$ and placed in a 37° C shaker-incubator with a platform rotating at a speed of 130-150 revolutions per minute. Incubation lasted 55 minutes. Similar preparations were made from sexually immature rats (3-4 weeks old, weighing 50-70 grams), except their fast lasted 6-12 hours; their intestinal sacs were cut 6 cm long; 6 ml Krebs-Ringer's bicarbonate buffer was used as the mucosal volume and 0.5 ml as the serosal volume.

Collection Of Samples

After incubation, each intestinal segment was dried on paper towel. Its content, representing the serosal volume, was poured into a glass vial. The incubating Krebs-Ringer's buffer of the flask constituted the mucosal volume.

Liquid Scintillation Counting

Radioactive measurements were taken of 0.30 ml aliquots of serosal and of mucosal solutions. The aliquots were diluted in a scintillation solvent that was composed of toluene, absolute alcohol, 2,5-diphenyloxadole (PPO) and p-bis 2-(5-phenyloxazolyl) (POPOP). A volume of 15 ml of the scintillation solvent was mixed with each 0.30 ml aliquot producing a sample consisting of 63.6 (v/v) toluene, 34.4% (v/v) ethanol, 2.0%(v/v) water, 0.40% (w/v) PPO and 0.01% (w/v) POPOP (0.01%). The liquid composition of the solution is well within the range of mutual solubility of toluene, ethanol and water (41).

A solubility test was run on the scintillation solvent and an aqueous sample. The test consists of counting volumes of $C^{14}$-aqueous samples (0.05

ml, 0.10 ml, 0.20 ml and 0.30 ml) diluted in 15 ml of scintillation solvent and comparing the different counts. Theoretically, the disintegration rate of 0.10 ml of sample should be twice that of 0.05 ml, half as large as that of 0.20 ml and a third of that of 0.30 ml. A difference of 0.48- 1.32% was noted between the DPM (disintegrations per minute) of 0.01 ml of sample and that of other samples (multiplied by their proper factor) due to solubility error produced by the non-uniformity of the sample in the scintillation solvent. Such a small factor would not alter the results.

Calculations

Expression of Amino Acid Concentration. Amino acid concentration was expressed in terms of the number of disintegrations per minute (DPM) in a 0.30 ml aliquot of the sample. The DPM represents a truer concentration figure than the counts per minute (CPM) in view of the fact that the CPM varies with the relatively variable efficiency of the counts. For example, it would be wrong to think that a solution of 25,015 counts per minute with a counting efficiency of 0.75 has the same $C^{14}$-amino acid concentration as a solution of 25,015 CPM with an efficiency of 0.80. Their respective disintegration rates are given by the following formula:

$$DPM = \frac{CPM^{sample} - CPM^{background}}{\text{counting efficiency}}, \text{ that is, } DPM = \frac{20,015 - 15}{0.75} = 26,666 \text{ DPM}$$

in the first sample and $DPM = \frac{20,015 - 15}{0.80} = 25,000$ DPM in the second sample. With a difference of the size of 1,666 DPM or 6.2%, it can be concluded the samples do not have the same amino acid concentration.

The background is a non-radioactive mixture of 97% scintillation solvent and 3% Krebs-Ringer's buffer. The purpose of subtracting a background from the count of the sample is to account for a few counts (usually

averaging 15 CPM) due to noises, quenching effects and other factors which are present in the counter.

The channel-ratio method gives the counting efficiency of the samples. This method uses the inverse proportionality of efficiency to the ratio of the B channel over that of the C channel of the counter. Efficiency is read as the ordinate of the graph shown in the appendix. An alternate and more accurate method of obtaining counting efficiencies is the internal standardization method. This involves the use of standard $C^{14}$-toluene of known disintegration rate. The closeness of the measurements from the two different methods is a function of the performance of the liquid scintillation counter. The instrument, after being tested for its performance, showed an average difference of 1.45% between the two efficiencies. Such a difference was due to mechanical errors in the performance of the counter added to pipetting errors. A further check on the performance of the counting apparatus consists of evaluating the counts. The repeatability of the counts for the same sample is a sign of good performance and proper operation in the machine. Following the steps outlined in a Packard Operating Manual (42), a sample was counted thirty times. 66.66% of the counts fell within the range of the average plus or minus the square root of the average. This is more than the minimum 66% that is required for good performance in the counter.

Expression of the Effects of Chemical Agents. Amino acid transfer from mucosa to serosa was expressed as the ratio of the amino acid concentration on the serosal side (S) over that on the mucosal side (M) at the end of a 55 minutes incubation period. Amino acid uptake in two different segments of the intestine could then be compared on the basis of their S/M ratio. Amino acid uptake averaged approximately 9% of the initial counts. About 1.5% was lost into the intestinal tissue.

The effects of insulin, sodium or D-glucose on amino acid absorption were expressed by subtracting algebraically the S/M ratio of control sacs from that of experimental sacs. A positive number, indicating a positive effect of a given physiochemical agent, resulted from such a subtraction when the absolute value of the experimental was greater than that of the control. There was a negative effect, designated by a negative value for the difference, when the absolute value of the experimental was less than that of the control. The ordinate axes of the graphs in Figures 1, 2, 3, 4, 5 and 6 represent scales of "effects on absorption." The zero lines on these scales were determined by the controls.

Because of unequal absorption rates in the three main parts of the small intestine and because of differences between individual organisms, these subtractions were carried out between experimental and control sacs of the same intestinal segment taken, whenever possible, from the same rat (duodenum, jejunum or ileum).

Histological Procedures

In order to study histological differences among the three main intestinal segments, 10 micron thick transverse sections of the small intestine of male rats were prepared. They were fixed in Bouin's fluid, stained with Harris' alum hematoxylin and counterstained with eosin Y. They were examined under a microscope provided with a calibrated micrometer.

The number of villi per $10_{\mu}$ transverse section was counted; the distance from the villi base to the internal border of the serosa was measured and the surface area of the villi was calculated using the formula

$$S = \pi D \left(h+\frac{D}{4}\right),$$

S: surface area

$\pi$: 3.1416

D: diameter of (cylindrical) villi

h: height of (cylindrical) villi.

The equation represents the lateral surface area of a cylinder ($\pi Dh$) plus the surface area of one base of the cylinder ($\pi\frac{D^2}{4}$).

# RESULTS

## Amino Acid Absorption along the Intestine

### Histological Considerations

A factor that was of great importance in the interpretation of the results was the non-uniformity of absorption along the intestine. A histological study conducted at the light microscope level (Table I) showed regional differences in total surface available for absorption along the length of the small intestine and in the thickness of the intestinal wall components of possible significance.

Structures Exposed to the Intestinal Lumen. According to the relation, transport rate = surface area x flux, at a given flux value, a larger exposed area makes for a more rapid transport rate (43). Since villi are responsible for increasing the number of epithelial cells exposed to the lumen (43), differences in their occurrence along the small intestine constitute a legitimate morphological basis for comparing transport rate in the duodenum, the jejunum and the ileum.

Villi are most numerous in the ileum, less numerous in the duodenum and fewest in the jejunum (Table I). A histological examination of the mucosa revealed a uniform distribution of the villi in the duodenum and the upper jejunum. In the ileum, clusters of villi were randomly interrupted with lymph nodules. Villi were generally taller and wider in the jejunum. The number, the height, the diameter of the villi and the surface area of the average villus were included in a comprehensive value: the mean surface area of villi per transverse section (Table I).

On the basis of surface area of villi alone, absorption would be expected to be higher in the duodenum and the jejunum and lower in the ileum (Table I).

Other structures that may cause differences in absorption rate along the intestine are the plicae circulares. They form in the submucosa, pass through the muscularis mucosae and extend to the mucosa. Their occurrence in the small intestine was not determined in this study.

Structures not Exposed to the Intestinal Lumen. The non-mucosal parts of the intestine, namely the muscularis and the tunica serosa, are at least passively involved in transfer as measured by the everted-sac technique. The thickness of these layers should influence transport rates in that passage through these layers should vary inversely with the thickness of the tissue. The average width of the muscularis plus the serosa is about 147 $\mu$ in the ileum, 158 $\mu$ in the jejunum and 163 $\mu$ in the duodenum (Table I). These numbers alone imply that passage through the layers that are not exposed to the lumen should increase slightly from duodenum to jejunum to ileum.

It is evident that the results obtained from the calculation of the mean surface area of villi per section and from the measurements of the width of the muscularis plus the serosa suggest that these two factors would be expected to have opposite effects on relative transport rates in the three regions of the intestine.

TABLE I

NUMBER AND DIMENSIONS OF STRUCTURES INVOLVED IN ABSORPTION IN INTESTINAL SEGMENTS

| | Duodenum | Jejunum | Ileum |
|---|---|---|---|
| Number of villi per cross-section | 29 | 26 | 37 |
| Number of sections counted for villi | 6 | 6 | 7 |
| Villi height | 389 μ | 393 μ | 296 μ |
| Villi diameter | 117 μ | 127 μ | 68 μ |
| Surface area of the average villus | 0.1537mm$^2$ | 0.1611mm$^2$ | 0.0671mm$^2$ |
| Number of villi measured | 15 | 14 | 17 |
| Mean surface area of villi per cross-section | 4.4595mm$^2$ | 4.3969mm$^2$ | 2.4824mm$^2$ |
| Average width of muscularis + serosa | 163 μ | 158 μ | 147 μ |
| Number of sections | 21 | 12 | 19 |

Regional Differences in Intestinal Absorption

In order to study apbsorption differences along the small intestine, a series of experiments using the everted-sac procedure were repeated with 16 cm segments. The incubating solution was a Krebs-Ringer's bicarbonate buffer containing L-alanine without insulin, glucose or any other physiological agent. It was found that, in general, within each main division of the intestine, the S/M ratios differed from each other by relatively close margins. But S/M values obtained from duodenal segments were markedly different from those of jejunal or ileal sacs (Table II).[1]

Absorption differences among selected 6 cm intestinal sacs were also studied. Measuring from the pyloric cecum, a series of sacs were made starting at a distance of 7-8 cm (second duodenal sac), 25-26 cm second jejunal sac) and 49-50 cm (second ileal sac).

[1]It was observed that the first duodenal and the last two ileal sacs were located in region of low amino acid absorption. Their S/M ratios are reported separately in Table II. The first duodenal sac showed the lowest S/M ratio. The S/M ratio in the lower ileum was lower than those of the jejunum and upper ileum. These facts were taken into account in the calculation of the effects of physiochemical agents on absorption by comparison between experimental and control results. An experimental from the first sac of the duodenum of a given rat, for example, was compared with a control taken from the upper duodenum of another rat of nearly equal weight and age. Experimental and control sacs from lower ileum were also compared with each other for the estimation of the effects of physiochemical agents on absorption.

TABLE II

VARIATIONS IN THE ABSORPTION OF L-ALANINE
ALONG THE SMALL INTESTINE OF A
SEXUALLY MATURE MALE RAT

| Intestinal parts | Positional order of intestinal sacs | Final S/M * |
|---|---|---|
| Duodenum | 1 | 0.29 |
| Duodenum | 2 | 0.51 |
| Duodenum | 3 | 0.59 |
| Jejunum | 4 | 0.70 |
| Jejunum | 5 | 0.59 |
| Jejunum | 6 | 0.74 |
| Jejunum | 7 | 0.66 |
| Jejunum | 8 | 0.88 |
| Ileum | 9 | 1.37 |
| Ileum | 10 | 0.89 |
| Ileum | 11 | 1.02 |
| Ileum | 12 | 1.37 |
| Ileum | 13 | 0.81 |
| Ileum | 14 | 1.18 |
| Ileum | 15 | 0.65 |
| Ileum | 16 | 0.54 |

* $S/M = \frac{\text{serosal } C^{14} \text{ concentration}}{\text{mucosal } C^{14} \text{ concentration}}$ ; initial S/M ratios were 0.

The results shown in Table III represent the mean values of the S/M ratios of these sacs. They indicate that amino acid transfer from mucosal side to serosal side is greater in the ileum and jejunum than the duodenum.

In an attempt to compare amino acid absorption between adult and sexually immature rat intestines, similar experiments were conducted with L-alanine using young male rats. The results followed the same trend as for the adults, namely that the absorption rate of L-alanine is higher in the ileum and the jejunum and lower in the duodenum(Table IV).

TABLE III

AMINO ACID ABSORPTION IN THE THREE MAIN SEGMENTS OF THE SMALL INTESTINE IN SEXUALLY MATURE RATS

| | Duodenum | Jejunum | Ileum |
|---|---|---|---|
| L-alanine | | | |
| Final* S/M ± S. E.** | 0.58 ± 0.04 | 0.76 ± 0.04 | 0.83 ± 0.04 |
| Number of experiments | 18 | 18 | 18 |
| Standard deviation | 0.11 | 0.18 | 0.26 |
| P (duodenum vs jejunum and/or ileum)*** | | $< 0.005$ | |
| L-lysine | | | |
| Final* S/M ± S. E. ** | 0.49 ± 0.05 | 0.63 ± 0.05 | 0.63 ± 0.05 |
| Number of experiments | 3 | 3 | 2 |
| Standard deviation | 0.003 | 0.16 | 0.04 |
| P (duodenum vs jejunum and/or ileum)*** | | $< 0.1$ | |
| α-aminoisobutyric acid | | | |
| Final* S/M** | 0.56 | 0.57 | 0.63 |
| Number of experiments | 1 | 1 | 1 |

* Initial S/M ratios were 0.

** $S/M \pm S.E. = \frac{\text{serosal } C^{14} \text{ concentration}}{\text{mucosal } C^{14} \text{ concentration}} \pm$ standard error.

*** P = significance of the difference between the sample means of the duodenum and those of the other two intestinal segments, based on the least-significant-difference test (44,45).

TABLE IV

ABSORPTION OF L-ALANINE IN THE THREE MAIN SEGMENTS OF THE SMALL INTESTINE OF SEXUALLY IMMATURE RATS

| | Duodenum | Jejunum | Ileum |
|---|---|---|---|
| Final* S/M ± S.E.** | 0.66 ± 0.08 | 0.89 ± 0.08 | 0.90 ± 0.08 |
| Number of experiments | 6 | 7 | 7 |
| Standard deviation | 0.10 | 0.19 | 0.29 |
| P (duodenum vs jejunum and/or ileum)*** | | 0.05 | |

*Initial S/M ratios were 0.

**S/M ± S.E. = $\frac{\text{serosal } C^{14} \text{ concentration}}{\text{mucosal } C^{14} \text{ concentration}}$ ± standard error.

***P = significance of the difference between the sample means of the duodenum and those of the other two intestinal segments, based on the least-significant-difference test (44,45).

## Effects of Insulin on L-Alanine Absorption

An important purpose of this study was to determine the dose-response effect for insulin with regard to L-alanine transfer through the intestinal wall.

Effects of Insulin on Absorption in Sexually Mature Rats. The effects of insulin on L-alanine absorption in the small intestine of sexually mature male rats vary from inhibition at concentrations in the neighborhood of 0.5 unit per 0.75 ml of serosal volume to stimulation at 3.0-3.5 units where maximum absorption is observed. At higher concentrations, the response is highly variable (Table V).

Figures 1, 2 and 3 represent graphs of the effects of insulin on L-alanine absorption versus its concentration. They show insulin effects in the duodenum, the jejunum and the ileum respectively.

Effects of Insulin on Absorption in Sexually Immature Rats. The graph of insulin effects versus its concentration in sexually immature rats has a similar shape to that of adult rats.[1] The main difference between the two kinds of graphs lies in the concentrations corresponding to positive effects of insulin. In the sexually immature male rats, the peak of the graph is displaced toward the left. Maximum effect of insulin in these rats is observed at a concentration of 2.25 units per 0.75 ml of serosal volume (Table VI, figures 4,5,6).

[1]Experiments were run using two or three different insulin concentrations for each segment of the intestine on the same day. They were repeated using the same insulin concentrations on different days. This procedure was generally followed when working with sexually immature rats as it was when working with adult rats.

TABLE V

EFFECTS OF INSULIN ON L-ALANINE ABSORPTION IN SEXUALLY MATURE MALE RATS

| Insulin concentration* | Effect ± S.E. (#)** in duodenum | Effect ± S.E. (#)** in jejunum | Effect ± S.E. (#)** in ileum |
|---|---|---|---|
| 0.1 | - 0.004 ± — (1) | - | - 0.28 ± 0.04 (3) |
| 0.5 | - 0.16 ± 0.04 (3) | - 0.07 ± 0.01 (5) | - 0.14 ± 0.04 (8) |
| 1.0 | - 0.14 ± 0.04 (4) | + 0.10 ± 0.01 (5) | - 0.08 ± 0.04 (7) |
| 1.5 | - 0.04 ± 0.04 (4) | - 0.10 ± 0.01 (5) | + 0.08 ± 0.04 (8) |
| 2.0 | - 0.09 ± 0.04 (4) | + 0.05 ± 0.01 (4) | - 0.04 ± 0.04 (8) |
| 2.5 | - 0.07 ± 0.04 (4) | - 0.08 ± 0.01 (6) | - 0.10 ± 0.04 (6) |
| 3.0 | + 0.08 ± 0.04 (4) | + 0.11 ± 0.01 (5) | + 0.12 ± 0.04 (8) |
| 3.5 | + 0.11 ± 0.04 (4) | + 0.14 ± 0.01 (4) | + 0.12 ± 0.04 (7) |
| 5.0 | - 0.12 ± 0.04 (4) | + 0.10 ± 0.01 (4) | - 0.06 ± 0.04 (8) |
| 10.0 | - 0.01 ± - (1) | - | - |
| Total number of experiments | 33 | 38 | 63 |
| F value | 4.76 | 2.71 | 5.74 |
| P*** | < 0.005 | < 0.05 | < 0.005 |

*Insulin (U/0.75cc) was placed on the serosal side of the intestine.

**The values of the effects of physiological agents on amino acid absorption were calculated by subtracting algebraically control S/M from experimental S/M.

S.E.= standard error.

(#): Numbers in parentheses indicate the numbers of experiments at each insulin concentration.

***P= significance of the difference between the sample means based on the everted beta distribution (46).

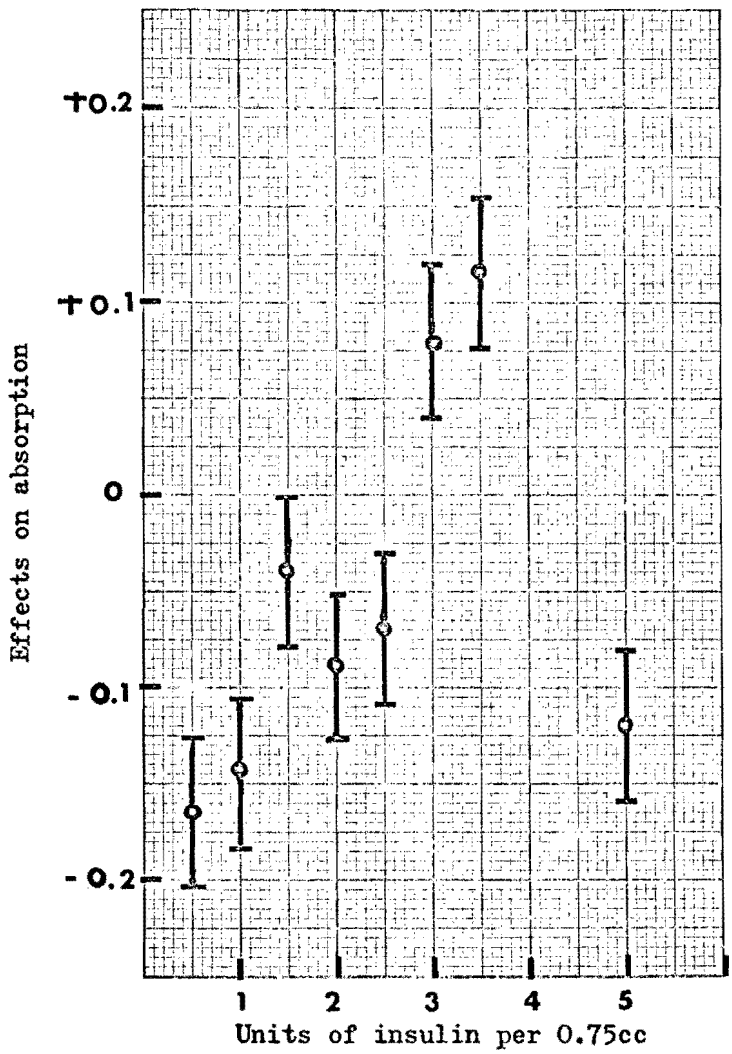


Figure 1. Insulin effects on L-alanine absorption in sexually mature male rats (duodenum).[1]

[1]The S/M values of the controls were arbitrarily assigned as the 0 point on the ordinate axis. The effects of insulin were calculated by subtracting algebraically control S/M from experimental S/M.

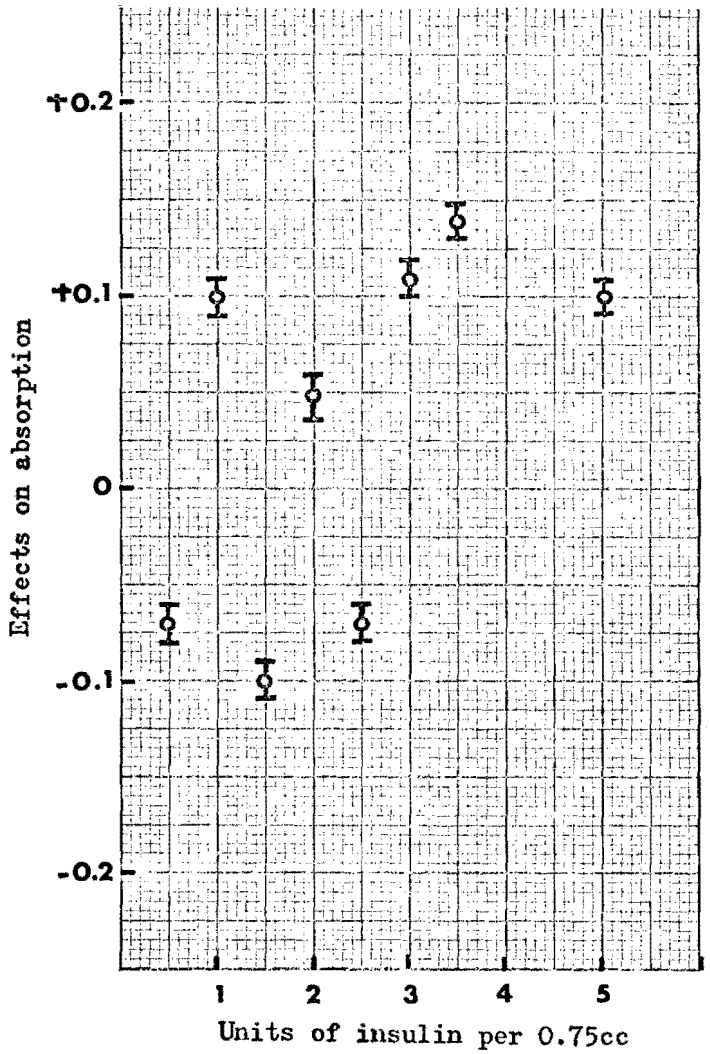


Figure 2. Insulin effects on L-alanine absorption in sexually mature male rats (jejunum).[1]

[1]The S/M values of the controls were arbitrarily assigned as the 0 point on the ordinate axis. The effects of insulin were calculated by subtracting algebraically control S/M from experimental S/M.

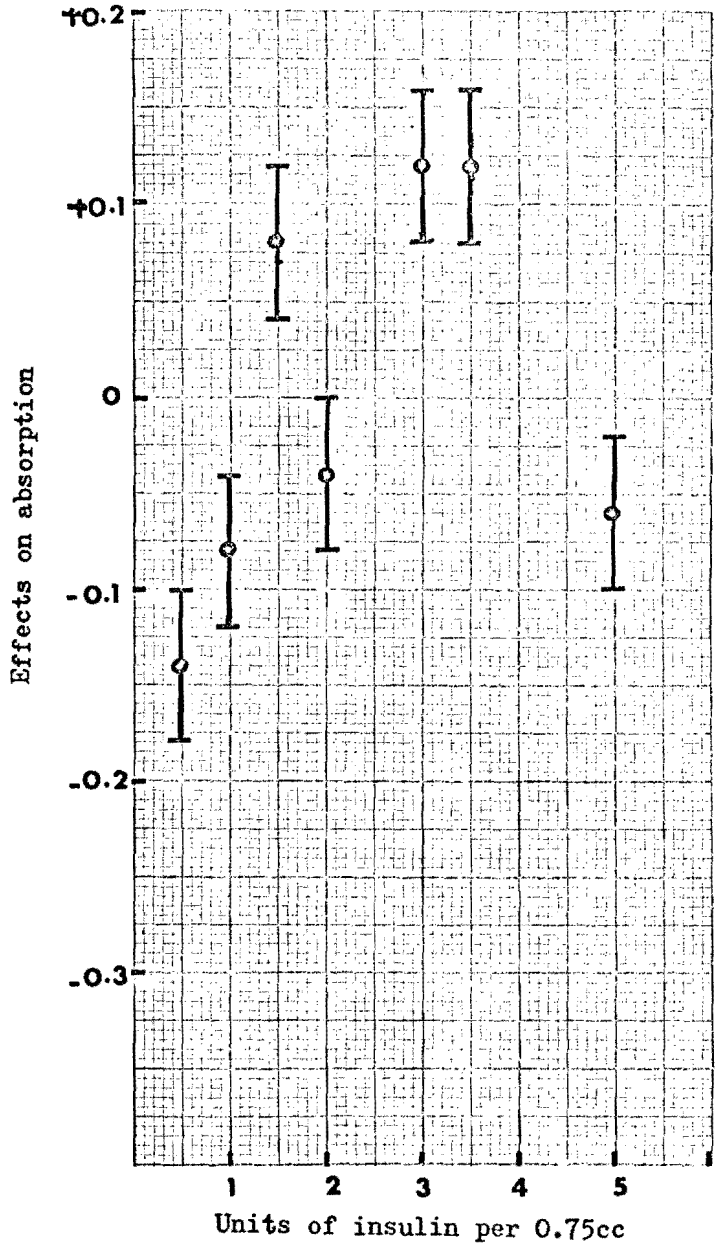


Figure 3. Insulin effects on L-alanine absorption in sexually mature male rats (ileum).[1]

[1]The S/M values of the controls were arbitrarily assigned as the 0 point on the ordinate axis. The effects of insulin were calculated by subtracting algebraically control S/M from experimental S/M.

TABLE VI

EFFECTS OF INSULIN ON L-ALANINE ABSORPTION IN SEXUALLY IMMATURE MALE RATS

| Insulin concentration * | Effect ± S. E. (#)** in duodenum | Effect ± S.E. (#)** in jejunum | Effect ± S.E. (#)** in ileum |
|---|---|---|---|
| 0.75 | - 0.13 ± 0.05 (3) | - 0.09 ± 0.06 (2) | - 0.26 ± 0.10 (3) |
| 1.50 | + 0.04 ± 0.05 (3) | + 0.13 ± 0.06 (3) | + 0.35 ± 0.10 (4) |
| 2.25 | + 0.19 ± 0.05 (3) | + 0.26 ± 0.06 (4) | + 0.17 ± 0.10 (5) |
| 3.00 | - 0.13 ± 0.05 (3) | - 0.04 ± 0.06 (3) | + 0.13 ± 0.10 (3) |
| 3.75 | - 0.19 ± 0.05 (2) | - 0.07 ± 0.06 (2) | - 0.10 ± 0.10 (5) |
| 4.50 | - 0.11 ± 0.05 (3) | - 0.11 ± 0.06 (5) | - 0.11 ± 0.10 (5) |
| 5.25 | - 0.17 ± 0.05 (2) | + 0.10 ± 0.06 (3) | - 0.22 ± 0.10 (4) |
| 7.25 | - 0.07 ± 0.05 (3) | + 0.13 ± 0.06 (4) | + 0.06 ± 0.10 (4) |
| Total number of experiments | 22 | 25 | 33 |
| F value | 5.93 | 4.09 | 4.00 |
| P*** | $< 0.005$ | $< 0.01$ | $< 0.005$ |

*Insulin (U/0.75cc) was placed on the serosal side of the intestine.

**The values of the effects of physiological agents on amino acid absorption were calculated by subtracting algebraically control S/M from experimental S/M.

S.E.= standard error.

(#): Numbers in parentheses indicate the numbers of experiments at each insulin concentration.

***P= significance of the difference between the sample means based on the everted beta distribution (46).

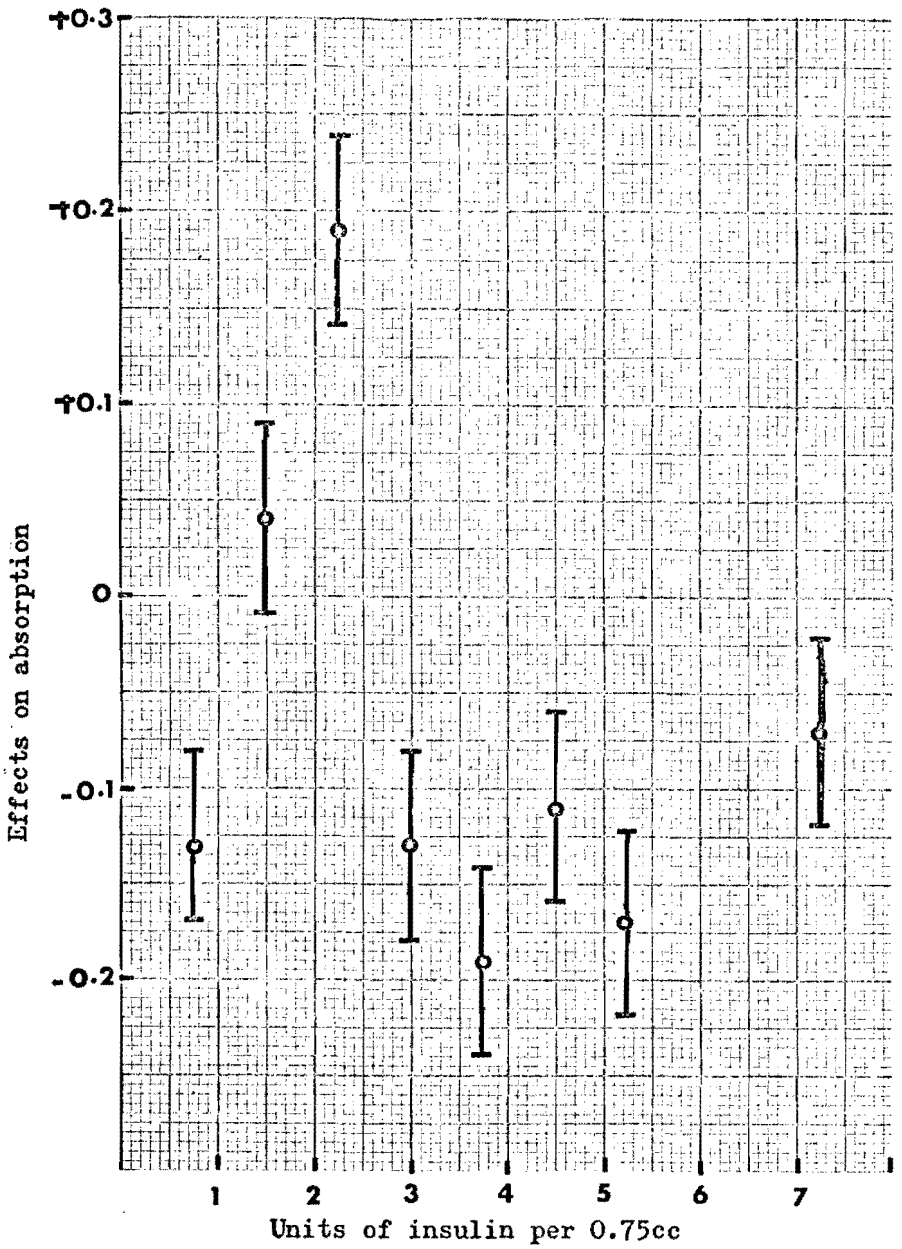


Figure 4. Insulin effects on L-alanine absorption in sexually immature male rats (duodenum).[1]

[1]The S/M values of the controls were arbitrarily assigned as the 0 point on the ordinate axis. The effects of insulin were calculated by subtracting algebraically control S/M from experimental S/M.

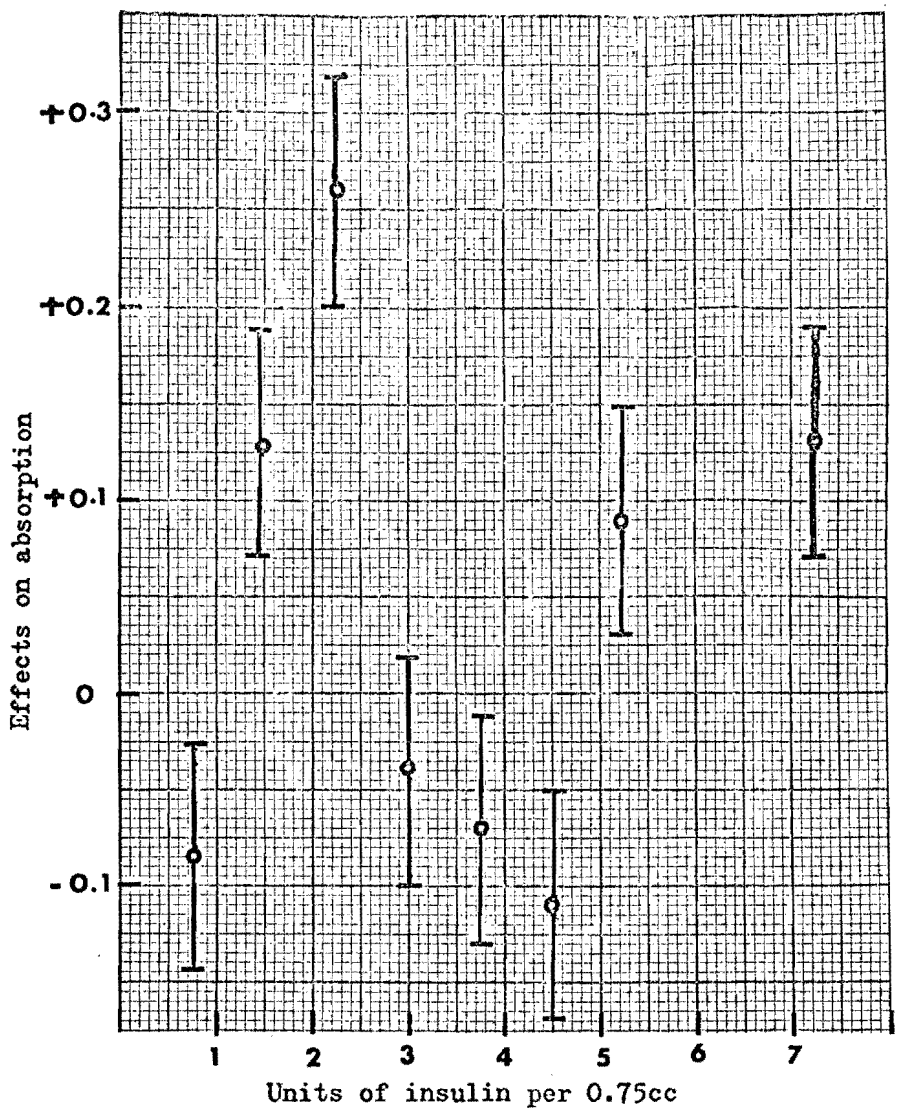


Figure 5. Insulin effects on L-alanine absorption in sexually immature male rats (jejunum).[1]

[1]The S/M values of the controls were arbitrarily assigned as the 0 point on the ordinate axis. The effects of insulin were calculated by subtracting algebraically control S/M from experimental S/M.

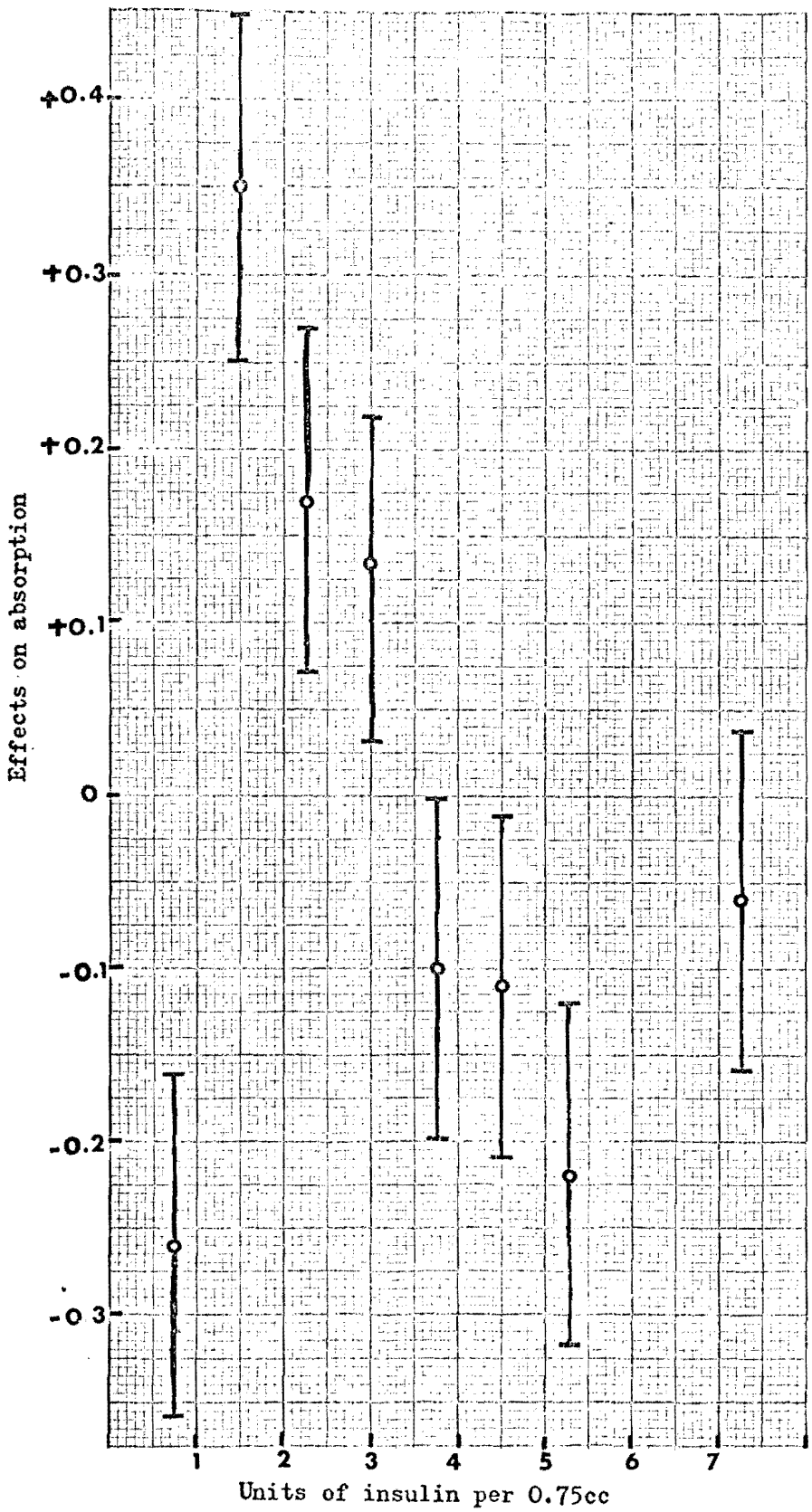


Figure 6. Insulin effects on L-alanine absorption in sexually immature male rats (ileum).

## L-Alanine Absorption in Sodium-Free Medium

A study was made of the effects of insulin and sodium on rates of amino acid transport.

The first part of the investigation consisted of comparing L-alanine transport through the intestine using a sodium-free Krebs-Ringer's bicarbonate in the sac and in the incubating medium to transport in a normal Krebs-Ringer's buffer medium. Experimental sacs taken from a given rat were compared to control sacs that were generally taken from the same rat. In most cases, the absence of sodium ions caused a decrease in L-alanine absorption (Table VII).

In the second part of the study, insulin was added to the serosal side and $Na^+$ was replaced with $K^+$ in the incubating Krebs-Ringer's solution. In the control flasks, a Krebs-Ringer's bicarbonate buffer containing $Na^+$ constituted the mucosal and serosal solutions and no insulin was present on the serosal side of the intestine. Insulin promoted L-alanine absorption in a sodium-free medium (Table VII).

TABLE VII

EFFECTS OF SODIUM AND/OR INSULIN ON L-ALANINE ABSORPTION IN SEXUALLY MATURE MALE RATS

| | Duodenum | Jejunum | Ileum |
|---|---|---|---|
| Without insulin | | | |
| $[Na^+]$ in mucosal and serosal solutions | 0 | 0 | 0 |
| Effect ± S.E. (#)* | - 0.17 ± 0.03 (4) | - 0.10 ± 0.04 (5) | - 0.10 ± 0.04 (8) |
| Standard deviation | 0.06 | 0.11 | 0.14 |
| F value | 4.33 | 4.16 | 10.00 |
| P (with vs without sodium ions)** | < 0.1 | < 0.1 | < 0.01 |
| With insulin | | | |
| $[Na^+]$ in mucosal and serosal solutions | 0 | 0 | 0 |
| Effect ± S.E. (#)* | + 0.20 ± 0.03 (4) | + 0.08 ± 0.04 (8) | + 0.14 ± 0.04 (9) |
| Standard deviation | 0.07 | 0.09 | 0.11 |
| F value | 39.93 | 10.09 | 13.89 |
| P (with vs without insulin)** | < 0.005 | < 0.01 | < 0.005 |

* The values of the effects of physiological agents on amino acid absorption were calculated by subtracting algebraically control S/M from experimental S/M.

S.E.= standard error.

(#): numbers in parentheses indicate the numbers of experiments.

**P= significance of the difference between the sample means based on the everted beta distribution (46).

D-Glucose and Amino Acid Absorption

The effect of D-glucose on L-lysine absorption was studied using a 2.0% (w/v) D-glucose Krebs-Ringer's bicarbonate buffer as the incubating medium. The mucosal and serosal Krebs-Ringer's solutions of control flasks contained no glucose and there was no insulin on the serosal side. Absorption in the presence of D-glucose and/or insulin was compared to absorption in these control flasks. In the presence of glucose, there was less absorption of L-lysine. The rate of absorption increased when (glucose being present in the incubating solution) insulin (3 U) was added to the serosal side of the intestine (Table VIII).

The same type of a study was repeated with a D-glucose concentration of 0.2% (w/v). Absorption in the presence of glucose and/or insulin was compared to absorption in a normal Krebs-Ringer's incubating medium (38). At a 0.2% glucose concentration, less L-alanine was absorbed. However, there was more uptake of the amino acid upon addition of insulin on the serosal side of the intestinal sac (Table IX).

Tables VIII and IX cannot be compared on a quantitative basis since a different amino acid was used in each case. These tables show that D-glucose exerts the same qualitative effect on two different amino acids.

TABLE VIII

EFFECTS OF D-GLUCOSE (2.0%) AND/OR INSULIN ON L-LYSINE ABSORPTION IN SEXUALLY MATURE MALE RATS

| | Duodenum | Jejunum | Ileum |
|---|---|---|---|
| Without insulin | | | |
| [D-glucose] in mucosal and serosal solutions | 2.0% (w/v) | 2.0% (w/v) | 2.0% (w/v) |
| Effect ± S.E. (#)* | - 0.15 ± -- (1) | - 0.06 ± 0.02 (3) | - 0.60 ± 0.10 (7) |
| Standard deviation | - | 0.02 | 0.73 |
| F value | - | 4.57 | 3.20 |
| P (with vs without D-glucose)** | - | < 0.1 | < 0.1 |
| With insulin | | | |
| [D-glucose] in mucosal and serosal solutions | 2.0% (w/v) | 2.0% (w/v) | 2.0% (w/v) |
| Effect ± S.E. (#)* | + 0.09 ± 0.04 (3) | + 0.12 ± 0.02 (3) | + 0.19 ± 0.10 (9) |
| Standard deviation | 0.07 | 0.06 | 0.29 |
| F value | - | 26.40 | 8.05 |
| P (with vs without insulin)** | - | < 0.01 | < 0.05 |

* The values of the effects of physiological agents on amino acid absorption were calculated by subtracting algebraically control S/M from experimental S/M.

S.E.= standard error.

(#): numbers in parentheses indicate the numbers of experiments.

**P= significance of the difference between the sample means based on the everted beta distribution (46).

TABLE IX

EFFECTS OF D-GLUCOSE (0.2%) AND/OR INSULIN ON L-ALANINE ABSORPTION IN SEXUALLY MATURE MALE RATS

| | Duodenum | Jejunum | Ileum |
|---|---|---|---|
| Without insulin | | | |
| [D-glucose ] in mucosal and serosal solutions | 0.2% (w/v) | 0.2% (w/v) | 0.2% (w/v) |
| Effect* (#)** | - 0.05 (1) | - 0.17 (1) | - 0.07 (1) |
| With insulin | | | |
| [D-glucose ] in mucosal and serosal solutions | 0.2% (w/v) | 0.2% (w/v) | 0.2% (w/v) |
| Effect* (#)** | + 0.01 (1) | + 0.17 (1) | + 0.17 (1) |

*The values of the effects of D-glucose were calculated by subtracting algebraically control S/M from experimental S/M.

**(#): numbers in parentheses indicate the numbers of experiments.

## Error Analysis

Q-Test. Before statistical values were estimated for small samples, a Q-test was run on outlying results that were suspected as having occurred as a consequence of some random error. The purpose of this test was to decide whether these border results should be maintained or rejected. This is a statistically valid test that is guaranteed with a 90% level of confidence (47). 14 out of 484 values (i. e. 2.9%) were rejected. The rejected values were excluded from the computation of sample means, variances and standard deviations.

Sources of Errors. The errors that were present in measuring amino acid concentrations by radioactive counting have been sufficiently dealt with (see pages 7, 8, and 9). Other types of errors that could cause some fluctuations in the results are personal errors such as volumetric errors leading to errors in concentrations.

## DISCUSSION

### On Differences in Absorption Potential among the Main Divisions of the Intestine

Booth (48) gives a general account on the location of absorption sites of nutrients along the small intestine. He drew a topographic chart which shows that different substances are best absorbed at different intestinal sites. His findings suggest that a given substance is not absorbed at the same rate in all parts of the intestine. The results presented in this paper show that amino acid absorption in the duodenum was slower than in the jejunum and the ileum. But no significant difference was noted between jejunal and ileal transport rates.

Absorption in the Duodenum. Compared to the rest of the small intestine, the duodenum is not a very active site of absorption. Few substances have been shown to be maximally absorbed in the duodenum *in vivo* and *in vitro* (49,50). Therefore, it is not surprising that the experiments showed the least absorption in this segment of the intestine.

Absorption in the Jejunum and the Ileum. Booth (48) remarks that absorption *in vivo* is in general less active in the ileum than the jejunum, notwithstanding the ability of the ileum to absorb efficiently substances that have escaped absorption in the proximal regions of the intestine. It would then seem that absorption *in vivo* should be less in the ileum than the jejunum partly because of its distal position. That would be one reason, besides others given by Booth, why "the site of maximum transport by an isolated segment of intestine *in vitro* may not correspond to its site of

maximum absorption *in vivo*."[1]

There is a surgical method that might permit one to determine if it is the proximal positon of the jejunum that causes it to absorb more than the ileum *in vivo*. The procedure involves the removal followed by the reversal of the jejunal and the ileal loops, the jeunum facing the cecum and becoming posterior to the ileum after surgery. If absorption is then maximal in the ileum, the hypothesis, namely that the positon of the post-duodenal segments causes one to absorb more than the other, is true.

The results presented in this paper show maximum *in vitro* absorption in the upper and middle ileum. Previous *in vitro* studies have shown that monoaminomonocarboxylic acids are best transported in the middle segments of the small intestine (39, 51, 52, 53, 54). If one considers the extreme segments of the adult Fischer rat intestine as being 18cm long each, the middle segments make up 40-60 cm covering the jejunum and the upper and middle ileum.

It turned out, as expressed in the data, that the ileum and the jejunum absorbed amino acids at a faster rate than the duodenum.

On the Effects of Insulin upon L-alanine Absorption

In reviewing the literature on intestinal absorption, one normally notices that two procedures are commonly followed: the Wilson-Wiseman everted-sac technique and the tissue accumulation method. The former gives results in terms of the amount of material that is transferred from the mucosal side to the serosal side of the intestine. The latter involves the measurement of substances accumulated in the tissue. Neither process, namely intestinal

[1]C.C. Booth, 1968, "Effect of location along the small intestine on absorption of nutrients," Handbook of Physiology, 3 (6): 1513-1514.

tissue accumulation or transfer from one side to the other, can be said to be intestinal absorption *per se* since transport is normally from the lumen of the intestine through the mucosa into the capillaries.

As insulin was placed on the serosal side of the intestine, it may have exerted its effects on transport in the serosa and possibly in the muscularis. Insulin, being a protein, may have not permeated through all the layers of the intestine. It is then likely that insulin has not come in contact with the mucosa. Fromm *et al.* circumvented the problem by stripping the small intestine of its serosa and its muscularis. Thus, they assured penetration of the insulin molecule to the basal surface of the mucosa (18). Their model represented a closer copy of *in vivo* situations than the everted sacs that were used in the present work. In their experiments, indeed, the transmural flux across the intestine could represent a close measure of villi absorption whereas the present investigation measured complete transfer of amino acids from the mucosal to the serosal side of the intestine.

Insulin, according to a standard textbook of medical physiology (55), increases amino acid transport at least into liver cells and possibly into other cells as well. Amino acid accumulation into diaphragm (13,14) and heart muscles (17) has been found to be stimulated by insulin (0.1 and 0.2U/ml). However, insulin (persumably 1U/ml) was reported to have no effect on amino acid flux across rat intestine *in vivo* (18).

The preceding work has not been aimed at a comparison of the effect of insulin on intestinal absorption at different concentration levels. In this paper, however, a range of concentrations varying from 0.1 to 5.0 units of insulin per 0.75ml of serosal solution was considered. Preparation of dose-response curves seemed valid in light of the variations in response to

different insulin doses. The graphs (see Figures 1, 2, 3, 4, 5, and 6) show that various insulin concentrations have different effects qualitatively and quantitatively.

Judging from the range of insulin concentrations used in the experiments, this paper describes more a pharmacological situation than a true physiological situation. These concentrations are 100 times (and more) the insulin levels of the plasma of normal rats. Insulin activity of plasma from fasting rats is believed to range from 5 to 30 milliunits per milliliter (56).

On Differences between Insulin Effects upon Adult and Immature Rat Intestine. A quantitative difference was observed between adult and sexually immature rats as to the effects of insulin on the duodenum and the ileum. The insulin concentration that corresponds to the maximum stimulatory effect on absorption is higher in sexually mature rats (see Figures 1, 2, 3, and Figures 4, 5, 6, and compare the peaks of the dose-response graphs for adult rats with those of young rats). At concentrations where a low response was observed in adults, the effects of insulin is relatively strong in sexually immature rats. It should be noted that between adult and 3-4 weeks old rats, there is not only a difference in sexual development, but also differences in factors such as body size and mass. The dry weight of a given length of intestine of an adult rat is greater than that of an equal length taken from a 3-4 weeks old (Table X). Quantitative differences observed in the responses of the intestine at the two ages may have something to do with such factors.

TABLE X

DRY WEICHTS OF INTESTINAL SEGMENTS OF A SEXUALLY MATURE AND A SEXUALLY IMMATURE RAT

| Segment source and length | Segment weight (216.7g mature rat) | Segment weight (56.2g young rat) |
|---|---|---|
| Duodenum-12cm | 0.198g | 0.175g |
| Jejunum-24cm | 0.404g | 0.283g |
| Ileum-36cm | 0.628g | 0.360g |
| Total weight of intestine* | 2.011g | 0.934g |

*The sum of the first 3 weight values for each animal is less than the total weight of the whole intestine because the 3 segments weighed do not represent the full length of the intestine.

On the Effect of Sodium upon L-Alanine Absorption

The results outlined in this paper are in accord with previous experiments which have been conducted on the role of sodium ions in intestinal absorption. It has been demonstrated on several occasions that $Na^+$ must be present for substantial amino acid transport across the small intestine (25, 26, 27). As indirect evidence of the role of sodium, ouabain, a known inhibitor of the sodium pump, reduces L-alanine uptake in rabbit ileum (57).

Effects of amino acids on sodium transport have also been studied. Adibi (28) has given evidence of an enhancing action of leucine on sodium absorption. Nevertheless, he did not see the possibility of a reciprocal action of sodium on amino acid uptake although a previous paper (27) had shown that leucine absorption was a sodium dependent process. Most workers have shown some sort of a facilitation of intestinal absorption of amino acids by sodium ions. Some authors (25, 26, 58) even see sodium as an essential factor in the active transport of amino acids.

The reason for low L-alanine absorption in the absence of sodium may be due to a lack of activity of the sodium pump. The sodium pump is a poorly understood mechanism. The term refers to the extrusion of sodium ions from cells against the electrochemical gradient. It is therefore a metabolic energy consuming system. This energy may be applied in the form of a $Na^+$ gradient, which is in turn formed at the expense of ATP (59). A similar conclusion was reached by Skou (60). Several workers (61, 62, 63) have observed a linear relationship between the rate of oxygen consumption and that of tubular reabsorption of sodium in kidneys.

According to Csaky (64), the energy-converting system, that is that part of the transport system which is responsible for the conversion of

chemical energy into pumping energy, requires intracellular sodium. But intracellular sodium is normally provided by diffusion of external sodium in vitro. Therefore, with no sodium in the incubating solution, intracellular sodium concentration is reduced (57, 64) and presumably the activity of the sodium pump decreases with consequent slowing down of active transport of amino acids.

On the Effects of Insulin upon L-Alanine Absorption in a Sodium-Free Medium

Insulin (3U), placed on the serosal side, increases L-alanine absorption whether or not sodium is present. This may be explained by the speculation that insulin is able to alter fine structures of responsive cells causing what Krahl (17) has called decompartmentation. Decompartmentation is a hypothetical sequence of intermolecular rearrangements that take place in the cell membranes which allows extracellular molecules to enter the cells.

On the Effect of D-Glucose upon Amino Acid Absorption

In considering the effect of D-glucose on intestinal transport of amino acids, one notices that all three possible results have been obtained from previous studies in the literature. Glucose, indeed, has been found to have an inhibitory effect (33, 35), a stimulatory effect (32, 34, 36) and no effect (37).

The results presented in this paper tend to support the view that D-glucose has an inhibitory effect on amino acid transfer across the small intestine. It was observed that the addition of D-glucose to the incubating medium caused a sizable decrease of L-lysine absorption. The measurement of D-glucose absorption was not a part of this project. However, there is much

evidence for the assumption that glucose is actively transported across the intestine (65, 66).

There exist possible interactions between the active transport of D-glucose and that of amino acids. It has been suggested that D-glucose inhibits amino acid absorption because the transport of the carbohydrate depends on the same carrier as amino acid transport (31). Such a carrier would be polyfunctional, provided with a series of separate binding sites (66). Each binding site is thought to be designed for a specific class of compounds, namely sugars, amino acids, electrolytes (31). Conversely, each substance has a certain affinity for its binding site which causes it to compete with other substances that depend also on the same site. This type of competition may occur between two sugars or two amino acids. Moreover, one substance, by combining with its binding site, may inhibit other binding sites of compounds of different groups by an allosteric mechanism (32, 68). Perhaps the effect of D-glucose on amino acid transport should be classified as allosteric.

This inhibitory effect on amino acid absorption is not peculiar to glucose only. Other actively transported carbohydrates, such as D-galactose, D-allose, 3-O-methyl-D-glucose, also cause intestinal transport of amino acids to decrease (31). D-fructose, L-sorbose and 2-deoxy-D-galactose, sugars which are not actively transported, have been reported to have no inhibitory effect on the absorption of certain amino acids including L-alanine and L-lysine (30, 31).

On the Effect of Insulin upon Amino Acid Absorption in the Presence of D-Glucose

The effect of D-glucose on amino acid absorption has received more attention in the literature than the combined effect of insulin and D-glucose. Kipnis and Noall (12) found that insulin (0.4U/ml), in the presence or the absence of D-glucose, induced a 3-5 fold increase of $^{\alpha}$-amino-isobutyric acid incorporation in the isolated diaphragm of the rat. Other papers have shown the independence of the stimulatory effect of insulin (0.1, 0.2, 0.5U/ml) on rat diaphragm from D-glucose (14, 69, 70). The results presented in this paper suggest that the small intestine, in the presence of D-glucose, respond to insulin in a similar fashion to the diaphragm.

The stimulatory effect of insulin is not understood. Several mechanisms, however, have been suggested. By the process of decompartmentation, some pores may have been enlarged or formed by molecular rearrangements in the cell membranes of the intestine. As a result, amino acid transport, in the presence or the absence of D-glucose, may have been made easier.

On Osmosis

Osmosis did not appear to be significant in these experiments on absorption. The final serosal and mucosal volumes were sometimes measured and compared with their respective initial volumes. Water absorption ranged from 0% to 0.5%.

Investigation on osmosis was limited in the course of this research work. However, an analysis of the osmotic conditions of the serosal and the mucosal volumes showed that the two solutions differed on the average by 0.66 micromole of HCl (the insulin diluent) per milliliter of the serosal

solution and $1.3 \times 10^{-3}$ micromole of the amino acid per milliliter on the mucosal side. It can be seen, by a simple subtraction, that the serosal side of the intestine was hypertonic to the mucosal side by 0.658 micromole of HCl. Such a minute concentration of hydrogen and chloride ions did not significantly offset the osmotic equilibrium and did not cause water flow from one side to the other.

# CONCLUSION

These experiments indicate that the rate of absorption of L-alanine, L-lysine and α-aminoisobutyric acid is greater in the ileum and the jejunum than the duodenum. However, amino acid absorption in all three intestinal segments showed generally a similar response to physiological agents such as insulin sodium and D-glucose.

A dose-response effect was determined for the pancreatic hormone, insulin. The graph of insulin effect versus its concentration on the serosal side followed similar patterns in the main divisions of the intestine: a rise, as the concentration of insulin increases, from inhibition to low stimulation to a maximum enhancing effect.

Amino acid absorption was inhibited in a sodium-free medium. It is suggested that such hindrance was caused by an inhibition of sodium pump activity on which amino acid transport is dependent.

Absorption of the amino acids L-alanine and L-lysine was inhibited by the presence of D-glucose in the incubating solution. This is thought to result from competition between amino acids and sugars for a common carrier.

It is to be hoped that more research involving autoradiography, electronmicroscopy and other sophisticated modern techniques can be made on the response of cell membranes to insulin in order to shed more light on the mechanism of insulin action. These techniques may also be important in providing more information on the nature of the sodium pump and on the interactions between sodium, carbohydrates and amino acids in transport processes.

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APPENDIX

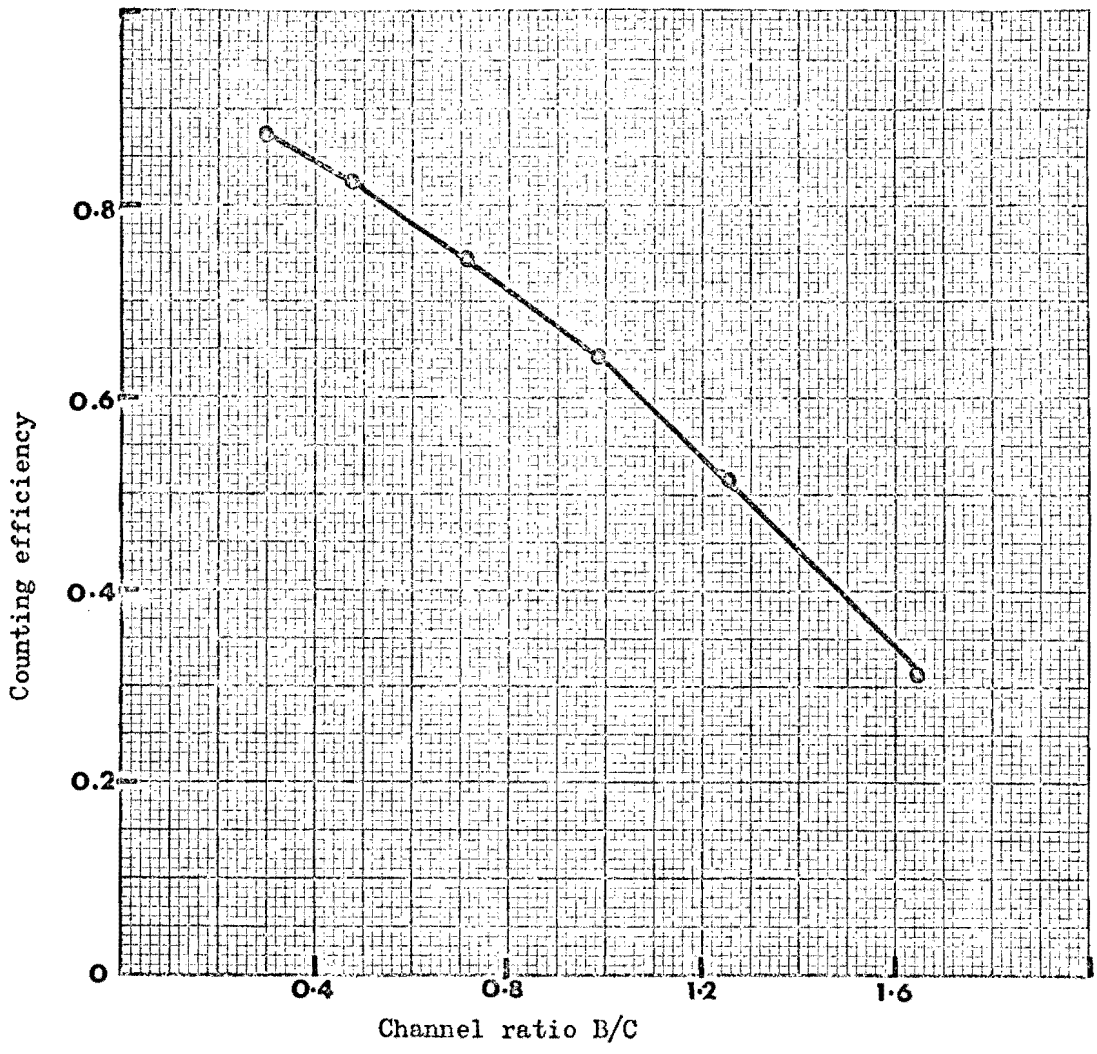


Figure 7. Liquid scintillation counting efficiency as a function of channel ratio.