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DEVELOPMENT OF A NEW DIGESTION TEST

BY

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A C K N O W L E D G M E N T
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The author wishes to express his gratitude to Miss Koharig Badjakian and Miss Lina Yaghi for their help in typing this manuscript.

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AN ABSTRACT OF THE THESIS OF

Yasser Yaghi for Master of Science in Biochemistry

Title: Development of a new digestion test.

1. Soluble starch was modified by partial methylation. The methyl group occupied the position 6 of the glucose molecule only.
2. The modified starch is digestable. Its break-down yields glucose and 6-O-methyl glucose.
3. 6-O-methyl glucose was found to be almost totally absorbed,passed through the liver unchanged and excreted in the urine.
4. The recovery of 6-O-methyl glucose after an oral administration of methylated starch was found to be more than 85% of the dose given.
5. Methylated starch may serve as a digestion test. It may be run simaltaneously with tests as D-Xylose and Phenyl-acetic oil in order to detect any mal-digestion or mal-absorption suspected.
6. When the methylated starch is given simultaneously with D-Xylose,the 5 hr urine contains at least 1.5 times methyl glucose as xylose (calculated as percentage of the dose.

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LIST OF ABBREVIATIONS

6-O-Meth. Glu.	6-O-Methyl Glucose
BAAN	Amino acid nitrogen in blood
C.B.D.	Common bile duct
Ci	Curie
G.I.	Gastro-Intestinal
HRS,hrs	Hours
IP	Intra-peritoneal
mCi	Millicurie
mmol	Millimole
ui	Microliter
N.G.	Naso-Gastric
PABA	Para Amino Benzoic Acid
S.D.	Standard deviation
mts	Minutes

CHAPTER I

INTRODUCTION

Many tests were proposed for assessing the digestive function of the pancreas. Such tests would be useful in the diagnosis of chronic diseases of the pancreas, and should be practical under conditions of ordinary hospital and office practice.

Tests to assess the digestion of carbohydrates, fats and proteins were suggested; a gelatin tolerance test was first proposed by West, Wilson and Eyles (1) for the purpose of diagnosing cystic-fibrosis of the pancreas in children. The test is based on the assumption that trypsin plays an important part in the hydrolysis of proteins, and that the rate of digestion of a protein meal can be estimated by the enusing elevation of the amino acid nitrogen in the blood (BAAN). These authors observed flat BAAN curves after ingestion of casein or gelatin, respectively in 7 and 2 children with cystic-fibrosis of the pancreas. When a casein hydrolysate or casein with pancreatin was administered to these children, a higher (up to normal) level of BAAN was produced. Anfanger and Heavenrich (2) utilized the gelatin tolerance test in 5 patients with this disease and confirmed the findings of West *et al* .They suggested that only

children with low BAAN can be reliably tested. In these cases the malabsorption of amino acids has to be ruled out as the cause of the low BAAN.

More recently, Woiski (3) reported similar observations with the gelatin test in four children with cystic fibrosis. The results of these investigators appear to be valid, although none of the reported series is large enough to permit statistical analysis.

Christensen and Shwachman (4) suggested the determination of a single amino acid, glycine in plasma, after ingesting gelatin, taking the advantage of the convenient method available for glycine (5), the high glycine content of gelatin (25,5 %) and the relatively slow disposition rateof this amino acid. Fifty patients were studied and the comparison of the fasting plasma glycine with the level 150 mts after gelatin feeding provided a simple diagnostic procedure, although some misleading results occured, mainly false negatives.

Another method was described (6,7) for measuring the degree of protein digestion and absorption by administering a test meal of protein labeled with radioactive iodine, following which the amount of radioactivity passed in the stools was determined. In normal control subjects les than 6 % of the ingested isotope was excreted in the feces, whereas in children with cystic-fibrosis of the

pancreas, 10-40 % (average 22.5 %) of the administered isotope was found in the stools. Morever in 2 of 4 children with this disease who were given pancreatin with the test meal, there was a 50 % fall in the amount of excreted isotope. In one patient in whom even after receiving pancreatin thare was still a 4o % excretion of the isotope in the feces, only 8 % was found when the test was repeated with hydrolyzed protein.

A similar method was used (8) as a diagnostic measure of pancreatic function in adults. The patients were given by mouth a test dose of protein labeled with radioactive iodine and the isotope was then measured in the urine. In the control normal group 62-90 % of the ingested isotope was found in the urine within 72 hours, whereas the fecal excretion varied from 0.5 - 4.8 %. In five patients with chronic pancreatic insufficiency, the urinary excretion for 72 hours was low, 20 - 56 %, and the fecal excretion high, 24 - 64 %. Pancreatin was administered to these patients and the urinary excretion of isotope fell to 6.5 - 36.2 %. In 2 of the 5 cases the administration of the pancreatin failed to produce any change.

A new test for exocrine pancreatic insufficiency was suggested by Imondi et al (9). In this procedure , a

synthetic peptide composed of an aromatic amino acid and a readily absorbable tracer group,para amino benzoic acid (PABA), was administered orally. In the presence of chymotrypsin, the peptide is split with the liberation of PABA which is absorbed from the gut, undergoes conjugation and is excreted in the urine . The amount of PABA in the urine is used as a measure of exocrine pancreatic function. This test proved to be more reliable than that described by Kaffarnick and Meyer-Bertenrath (10), in which fluorescein diesters are administered orally. These substrates are hydrophobic and require both bile and pancreatic lipase for splitting.

An extensive study has been reported (11) of methods for determining fecal trypsin by the digestion of x-ray film, particularly with reference to the effect of various techniques used, and to the use of a soybean trypsin inhibitor, in order to identify the protease of tryptic origin separately from that produced by bacteria. (About 28 % of normal feces have a gelatinase produced by bacteria.) A positive result at birth was obtained in only 76 % of meconium specimens which increased to 92 % in the post meconium specimens, as well as in other stools up to the age of five, over 9000 tests were performed on over 900 specimens from 216 children.

Having in mind the studies reported by Grossman (12), using dogs having pancreatic exclusion, and those reported by Ventzke et al.(13), suggesting the measurement of fecal trypsin and chymotrypsin for detection of pancreatic insufficiency. Haverback et al.(14), and Amman et al.(15), confirmed these results stressing on measuring the fecal chymotrypsin as a useful test in the diagnosis of pancreatic diseases. In later studies, Amman et al.(16) suggested the use of fecal chymotrypsin assays as screening methods for chronic pancreatitis and cancer of the head of the pancreas.

As for the study of the digestion of fatty acids, a method was proposed for the determination of fatty acids in dried feces, a tedious process, and one which is somewhat inaccurate if applied to pathological feces. Most of the methods for the determination of fatty acids in the moist feces are reliable, but require a longtime. A method has been proposed by Van der Kaner (17) for the determination of fatty acids in moist feces, which is speedy (requiring only 30 - 45 min.) convenient and accurate.

Another absorption-digestion test for lipids was suggested by Stanley and Thannhauser(18,19) ^{131}I triolein absorption test, but it showed many sources of errors in practice (2o,21,22,23,24).

More recently, our lab described a procedure which may serve as a useful simple clinical test for fat digestion- absorption.This test depends on feeding phenyl 1 - ^{14}C acetic acid incorporated into a triglyceride, to subjects and measuring the excretion of the phenyl acetate in a 5 hour urine collection (25,26).

While convenient tests are available to assess the digestion of fatty acids and proteins, there are no simple ways for measuring the digestion of carbohydrates. Several authors suggest that starch might be better than gelatin as a test material for this purpose, because of the weak amylolytic properties of ptyalin, the short duration of its action, and the presence of amylolytic enzymes in the succus entericus. The digestion of starch depends on the pancreatic amylase to a much greater degree than the digestion of gelatin depends on trypsin. With these considerations in mind a starch tolerance test was developed by Theodore et al(27,28), which had been proved to be unreliable, with many false positive and false negative results.

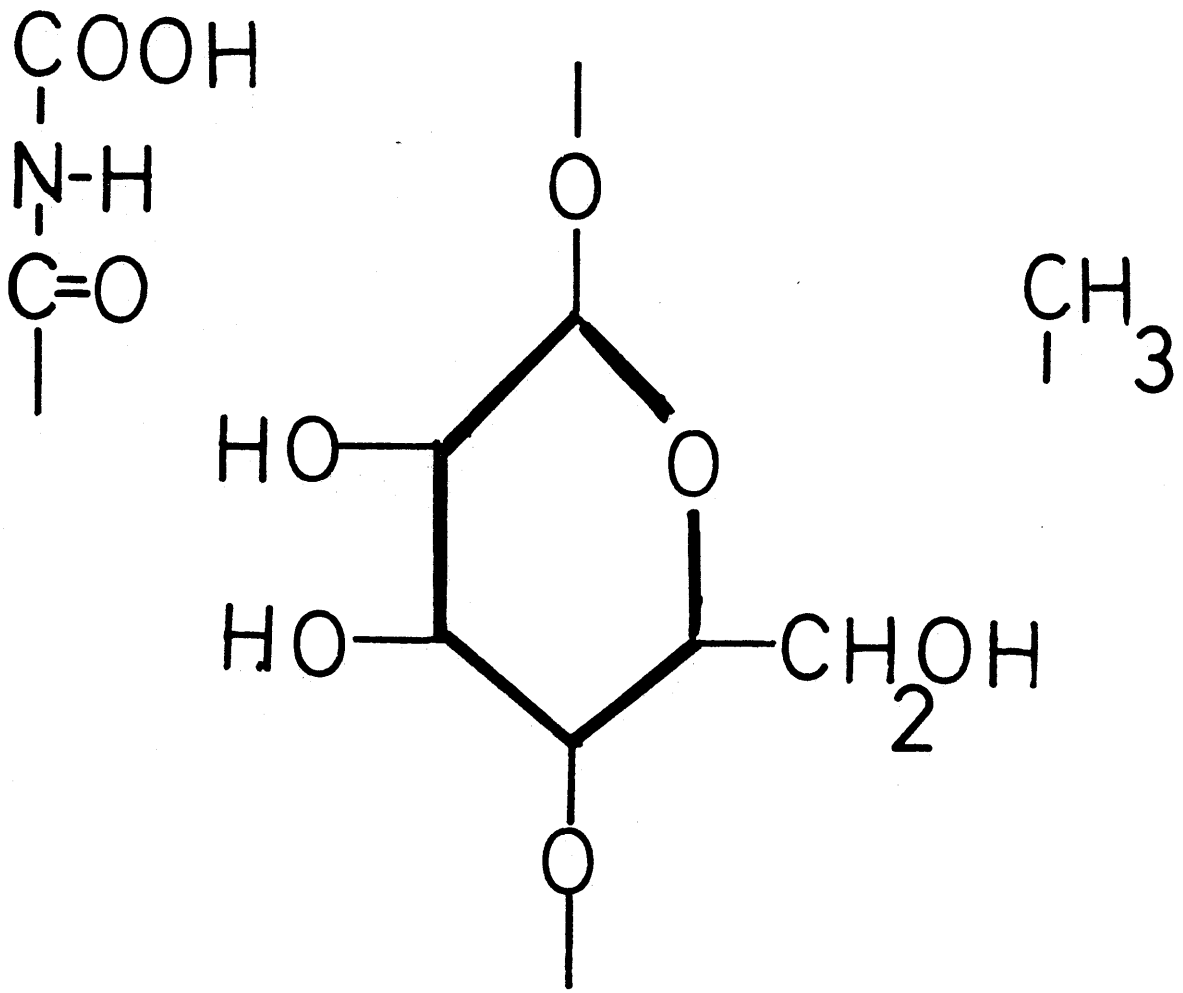


FIGURE 1. THE FREE HYDROXYL GROUPS OF GLUCOSE.
While CNBr reacts with 3 and 4 groups
the methyl sulfate occupy position 6.

Scope of the Investigation:

In the present study we describe a method to modify starch in such a way as to :

1 - retain its digestability with the pancreatic amylases .
2 - produce upon digestion an absorbable non metabolizable compound.
3 - This compound has to be non-toxic, easily excreted in the urine and easily detectable.
4 - I dealy such a substance or its determination should not interfere with other digestion or absorbtion tests such as the D-Xylose test so ti would be possible to run the two tests simaltanuosly.

Attempts were made to methylate the starch partialy, mainly on the C6 of the glucose, so that the breakdown of starch will yield 6-o- methyl Glucose which was found to be absorbed, not metabolized, and nearly totaly excreted and measured in urine.

Taking these facts into consideration, the 6-o-methyl Glucose test may serve as a chemical test in the diagnosis of the maldigestions due to pancreatic insufficiency and chronic pancreatic diseases.

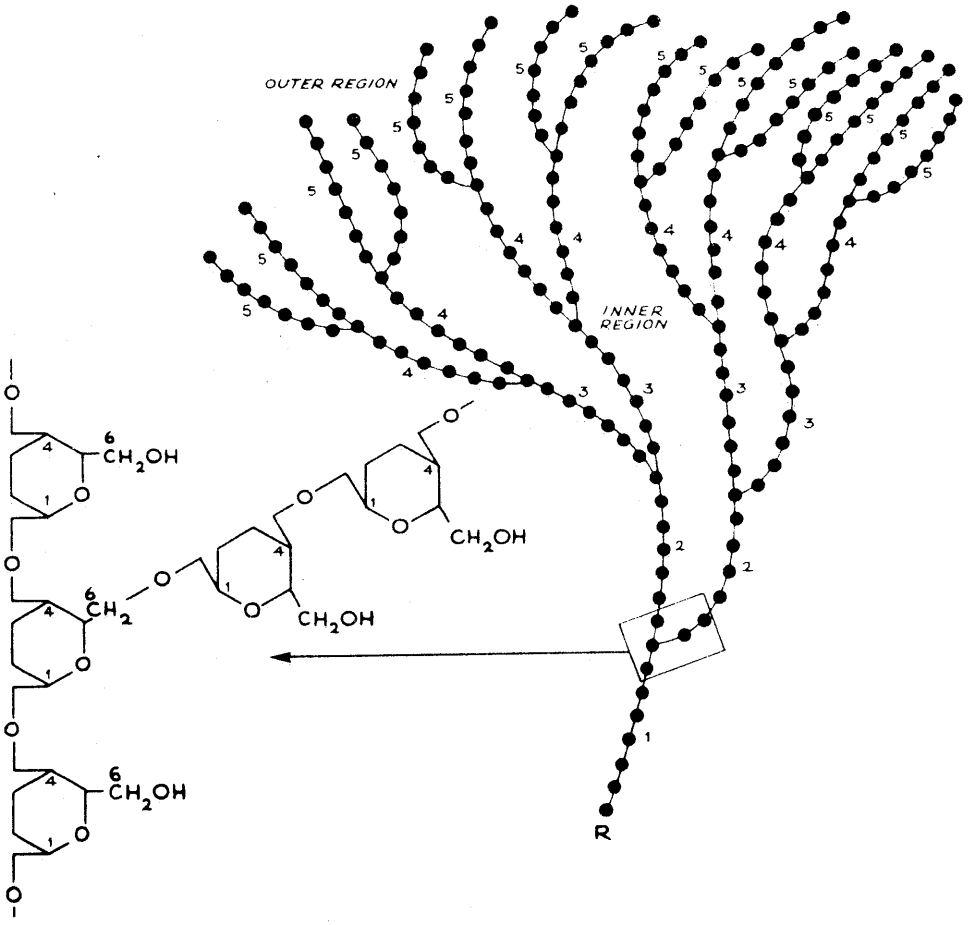


FIGURE 2. STARCH MOLECULE: The glucose residues are shown united by 1:4 or 1:6 linkages.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Non Radioactive Chemicals

a: Carbohydrates:

The soluble starch was a product of Mallinckrodt and had a maximum limit of ash impurities of 0.3%. While the Arrow Root starch (St. Vincent)was purchased from Amend Drug and Chemical Company,incorp.

D- Glucose,D(-)Ribose and Maltose were obtained from BDH Chemicals Ltd and D(+)Xylose from Sigma Chemical Company.

The 6-O-methyl- α ,β-D-Glucose and Mono and di-methyl Glucosides were a product of Supelco,Inc.

b: Reagents:

NN-Di-methyl-p-phenyldiamine and p-Anisidine were purchased from BDH Chemical Ltd, phenol(crystals) from J.T.Baker Chemical Co. and O-Toluidine from Sigma Chemical Company.Para-dimethyl-amino-benzaldehyde , (p-DMBA) was a product of Fisher Scientific Company.

c: Others:

Methyl Sulphate was obtained from Eastman Kodak Company and N,N-Di-methylformamide from Merck-Schuchardt.

Cyanogen Bromide was purchased from 2 sources: Eastman Kodak Company and Fluka AG.

Methyl Cyanide (Acetonitile) was obtained from Hopkin and Williams Ltd.

2. Radioactive Materials

Di(^{14}C) methyl Sulphate: activity 10-30 mCi/mmol, Di-(^{3}H) methyl Sulphate: activity 50-150 mCi/mmol,(2-^{3}H) Glycine; activity 20 mCi/mmol , L- (4,5-^{3}H) Leucine, activity 48 Ci/mmol,L-(4,5-^{3}H) Lysine monohydrochloride with activity of 40 Ci/mmol, were obtained from The Radiochemical Center, Amersham, Buckinghamshire, England.

Standard Tritiated H_2O of Radioactivity 2.156×10 ± 1% dpm, was purchased from Packard Instrument Company, Inc. U.S.A.

B. Methods

a. Dialysis and Methylation:

To remove small molecules, starch was dissolved in water and dialized 12 hours against water. The water was changed every 2 hours. The starch was then precipitated with five volumes of alcohol and dried in vacuo

over Phosphorous Pentoxide. 20 mg. of the dialized precipitated starch was suspended in 1 ml of acetone with continuous stirring. 40 ul di-methyl Sulphate and 80 ul of 30 % NaOH(in water) were added and vigorous stirring was maintained for 3 hours. The reaction product was precipitated with five volumes of alcohol and the precipitate dried in vacuo.

b. Acid Hydrolysis and Chromatography:

A fraction of the methylated starch was hydrolized with 1 N Sulphuric acid in a boiling water bath for 3 hours, then neutralized with 0.58 N Barium hydroxide. Fractions of the supernatant were run on a pre coated Silica Gel, Thin layer chromatography plate (Merck), 60F-254, and KIF Silica Gel 80 A (Whatman, Inc.). Samples of Glucose,Xylose,Maltose, Ribose , 6-O-methyl Glucose and mono and di-methyl Glucose were simultaneously run as standards. The solvents used (31) were; ethyl acetate: Acetic acid: Methanol: Water , 12:3:3:2 (v/v), and Propanol: Water, 8.5:1.5(v/v).

The spots were visualized using the following reagents;

- Phenol- Sulphuric Acid.(34).

- p-Anisidine.(32).
- NN-Di-methyl-p-phenyldiamine.(33).

c. Feeding Rats and Collecting Urine:

Wistar strain female albino rats were bought through the animal house of AUB, and maintained on the basic pellet diet. The labelled methylated starch was fed through a N.G. tubes.24 hours urines were collected in metabolic cages (Maryland plastics, Inc, U.S.A.)

Labelled methylated hydrolized starch was fed the same way, and the urines were collected again over 24 hours.

d. Assay for D-Xylose :

D-Xylose was measured in urine using the method of Goodwin (30).

e. Surgical Techniques :

Rats were anesthesized by the I.P. injections of 1.2 mg of Nembutal per Kg of body weight. The abdomen was opened through a midline incision. The common bile duct was ligated with a 2-0 silk suture. Steatorrhea occured. 48 hours after ligation, the rats were killed and bile duct obstruction was confirmed by autopsy.

Sham operations were also performed as control operations.

f. Experiments on humans :

Ten healthy human volunteers were tested. Their ages ranged between 21 and 65 years. Four of them were medical students and one was a faculty member, while the other five were patients from the A.U.H., all were free of any G.I. disease, and were on a regular diet until the evening priorto testing.

Five g of D-Xylose in 250 ml of water were given in addition to the labelled methylated starch. Two hour fractions of urine were collected throughout for 16 hours. The collection was continued for 24 hrs.

g. Measurement of Radioactivity :

Twenty ml of Bray's (29) solution were added to all the samples to be counted, and left for one hour in the dark to eliminate any photofluorescence. The samples were counted in a Packard Liquid Scintillation. Counts were corrected by the internal standards method.An efficiency of 80 % for C^{14} and 79 % for H^3 was usually obtained.

h. Spectrophotometry :

Spectra were taken using Perkin-Elmer 202 recording Spectrophotometer .

CHAPTER III

RESULTS

1. Methylation and Chromatography

a. The movement of fractions of methylated hydrolized starch on TLC plate run simultaneously with authentic samples of glucose, maltose, xylose, ribose, and mono-and dimethyl glucoses are shown in table I as R_g values calculated relative to the mobility of standard glucose.

From the table it is clear that in the methylated starch hydrolysate only glucose and 6-0-methyl glucose could be detected. Showing that the methylation of starch occured mainly on the 6 position.

b. Using our method 25 to 35 per cent of the radioactivity in Me_2SO_4 was incorporated into large molecular weight polymers.

c. Fractions of methylated labeled hydrolized starch were run on a TLC plate simultaneously with samples of glucose, hydrolized starch, and 6-O- methyl glucose. The results of this method showed that 95.82 per cent of the tritiated methyl was bound to the C6 of glucose.

2. Experiments on Rats

. Tritiated methylated starch was fed to 7 female rats

recovery of the tritiated material was 86.919 per cent $\pm$6.325 (S.D.) of the total counts administered.

TABLE I

Chromatographic separation of sugars with the solvent ethyl acetate: acetic acid: methanol: water, 12:3:3:2 (v/v).

Sugar	R_g
Glucose	1.0
Methylated Hydrolized Starch	1.0, 1.4
Xylose	1.5
Maltose	0.6
Ribose	1.3
2-0-methyl glucose	1.6
3-0-methyl glucose	1.5
4-0-methyl glucose	1.5
6-0-methyl glucose	1.4
2,4-di-methyl glucose	2.0
3,4-di- methyl glucose	2.0
4,6-di-methyl glucose	1.8
2,3,4,6-tetra-methyl glucose	2.3

R_g: cm sugar moved on plate per cm glucose moved on plate.

TABLE II

Excretion of Radioactivity in Urine of Rats fed methylated tritiated starch.

Animal No.	per cent excretion in 1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	Total Excretion 3 days
1	40.10	4.79	4.24	49.13
2	54.10	2.51	0.68	57.29
3	43.90	4.16	0.68	48.74
4	40.90	4.17	0.92	45.99
5	50.59	0.26	3.20	54.05
6	52.98	4.71	3.20	60.89
7	51.41	5.51	1.10	58.02

See results section 2 for details.

TABLE III

Excretion of Radioactivity in Urine of Rats fed methylated tritiated hydrolized starch.

Animal No.	per cent excretion in 1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	Total Excretion 3 days
1	52.00	4.80	0.15	56.95
2	51.00	5.90	1.10	58.00
3	48.01	0.35	0.10	48.46
mean	50.33 $\pm$2.08	3.68 $\pm$2.94	0.45 $\pm$0.56	54.47 $\pm$5.24

See results section 2 for details.

TABLE IV

Excretion of Radioactivity in Urine of Rats, with artificial common bile duct obstruction, fed methylated tritiated starch.

	per cent excretion within 24 hours
Rat with C.B.D. Obstruction	49.71
Rat with C.B.D. Obstruction	52.33
Rat had Sham Operation	52.40
Rat had Anesthesia	56.25
Control Rat	54.54
Control Rat	51.90

See results section 2 for details.

lucose and 3-0-methyl glucose were essentially the same. he absorption of 6-0-methyl glucose was about one third hat of glucose. In another study, Csaky (50) proved that -0-methyl glucose failed to transport against a concentra- ion gradient in intestinal loops.

When our labeled material was fed to the rats, 53.4 er cent ± 5.6 (S.D.) of it was recovered in the urine Table 2). While 86.9 per cent ± 6.2 (S.D.) of the mate- ial was recovered when fed to healthy humans. (Table 5). he difference in results may be due to the inconvenient nd unsatisfactory method used to collect rats urines, which ermits loosing urine during collection. This was totally ontrolled when human urine was collected.

Hydrolized labeled methylated starch was fed in the ame manner to rats and the recovery was 54.4 per cent .2 (S.D.) of the total amount administered. Nearly the ame as the methylated starch.

Experiments on the rats showing equal excretion of in- act and hydrolized methyl starch in three days , lead to he conclusion that our methylated starch is essentialy ompletely hydrolized. The better recovery of the methyla- ed starch in urine in humans, shows that more than 85 per ent of the test dose is excreted within 24 hours. Fig. 3.

A logarithmic plot of urinary excretion vs time shows a half life of the compound in the body of approximately 1.7 hours. Fig. 4.

As the digestion of the methylated starch appears to be essentialy complete, the time study of the urinary and blood 6-0-methyl glucose following oral administration of methylated starch, yields considerable information as to the digestive ability of the gastrointestinal tract. Healthy humans would excrete more than fifty per cent of the labeled 6-0-methyl glucose within the first five hours. While those having maldigestion problems are expected to have a lower initial excretion rate of the labeled material.

Intestinal maldigestion may be due to any chronic disease of the pancreas that would affect the excretion of the amylase.

This assumption is justified since it has been known that 6-0-methyl glucose is not metabolised, passes through the liver unchanged, and can be recovered from the urine. Unfortunately the testing of the usefullness of the methylated starch as a specific diagnostic test will have to await the collection of data on a number of patients with various diseases.

The D-Xylose test which has been for a long time the test of choice for passive absorption from the intestines,

may be run simultaneously with our test and the urine is collected over the first 5 hours after the ingestion of the two test materials, xylose and 6-0-methyl glucose. Our experiments show that xylose has initialy a much higher rate of excretion than 6-0-methyl glucose. Since the later is to be digested before it is absorbed. The results of both may give a wide clinical and physiological view about the digestion and absorption in the intestinal tract.(52, 53, 54).

Table 6 shows the mean and S.D. values for the excretion of D-Xylose and 6-0-methyl glucose. From the table it is clear that at 5 hours even if two S.D. from the mean is considered (so as to include 96 per cent of the population), 6-0-methyl glucose should be at least 1.5 fold the D-Xylose (as percentage of dose excreted). When the 6-0-methyl glucose is less than that value, maldigestion should be suspected.

When a -arge number of patients with malabsorption are tested, it may become possible to find other indicies for diagnosing maldigestion.

It is possible to run our test simultaneously with the fat absorption test developed in our lab in addition to the D-Xylose test. This will involve a single mixed oral dose and a single 5 hour urine collection.

As a battery the absolute results of the three tests as well as the ratios obtained could be extremely useful in differential diagnosis.

TABLE VI

The mean and S.D. values of three healthy volunteers, for the excretion of D-Xylose and 6-O-methyl glucose.

test achieved	per cent of dose excreted in the indicated period			
	2 hrs.	4 hrs	5 hrs	10 hrs
D-Xylose	9.85 ±0.35	18.35 ±0.70	22.42 ±1.68	36.93 ±0.66
6 O-methyl glucose	11.37 ±6.75	37.20 ±5.89	48.10 ±5.80	78.50 ±3.32

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