Metabolic Studies with 3-Methyl Glucose

1. ITS FATE IN THE ANIMAL BODY

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The balance of available evidence suggests that in glycogen and starch the hydroxyl groups in position 3 of the glucose residues are free. It therefore was possible that glucose carrying a substituent at position 3 might, in the animal body or elsewhere, be converted to a methyl derivative of glycogen. Many years ago unpublished preliminary experiments by one of us (F.G.Y.) indicated that 3-methyl glucose was not appreciably converted to glycogen in the liver or the muscles of the rabbit. This failure of the animal to utilize 3-methyl glucose to an appreciable degree, although it is a simple sugar derivative which closely resembles glucose in many chemical and physical properties, has been further investigated. A preliminary account of the results obtained has been recorded (Campbell, 1949).

The present paper describes experiments with the intact animal, while the following one (Campbell, 1952) is concerned with investigations *in vitro*.

EXPERIMENTAL

Synthesis, properties and estimation of 3-methyl D-*glucopyranose*

The synthesis from D-glucose followed that generally employed (Irvine & Scott, 1913; Levene & Meyer, 1922) in which D-glucose is converted to 1:2–5:6-di*iso*propylidene-D-glucofuranose, the only free OH group of which is then methylated and the *iso*propylidene groups removed by acid hydrolysis. After neutralization of the solution and evaporation to dryness the 3-methyl glucose is obtained as a white crystalline material by crystallization from methanol containing a little acetone. Di*iso*propylidene-glucose was prepared by the method of Bell (1935), although the procedure devised by Raymond & Levene (1929) for the separation of the di*iso*propylidene from the monopropylidene derivative, in which the former is extracted with boiling light petroleum (80–100°), was found to be a useful simplification of the method. From 100 g. of D-glucose about 25 g. of 3-methyl glucose were obtained.

An aqueous solution of the sugar had $[\alpha]_D^{20°}$ of $+98{\cdot}0° \pm 0{\cdot}1$ falling to $+58{\cdot}5° \pm 0{\cdot}1$. The corrected melting point was 157·5°. With Benedict's (1926) quantitative copper reagent the reducing power was 45% of that of glucose and approximately 80% with the Hagedorn & Jensen (1923*a*, *b*) $K_3Fe(CN)_6$ reagent. 3-Methyl glucose forms an osazone which has a characteristic crystalline formation which, although similar to that of glucosazone, can be differentiated from the latter under the microscope. A solution of 3-methyl glucose is not fermented by ordinary baker's yeast.

The content of 3-methyl glucose in pure solution and in tissue fluids containing the sugar was determined in two ways: (*a*) by the method of Zeisel (1885), with the volumetric procedure due to Viebök & Brecher (1930), in which the methoxyl content of the solution is determined; the procedure adopted followed that described by Elek (1939) with only minor modifications; (*b*) by removal of the glucose by means of yeast fermentation, and estimation of the residual reducing power of the solution. This method is based on that of Young (1934) and for plasma is as follows: to 2 ml. $ZnSO_4$ solution (1·8%) is added 1 ml. plasma followed by 2 ml. 0·085 N-NaOH. After 3 min. in a boiling-water bath the precipitate is filtered into a 30 ml. beaker and washed with 3 × 2 ml. hot distilled water. The beaker is placed in an oven at 37° for 15 min., 2 ml. yeast suspension, prepared according to the method of Somogyi (1927) at room temperature, are added and the whole incubated at 28° for 30 min. During this time the beakers are covered with watch glasses and are occasionally stirred. The yeast is then filtered off on a sintered-glass funnel (Jena 4G) and washed three times with 2 ml. distilled water. The filtrate is made up to 25 ml. with distilled water and suitable samples are taken for Hagedorn & Jensen estimations. The reducing value of the yeast suspension and the non-sugar reducing substances in the plasma is allowed for by a control estimation on a sample of plasma which does not contain 3-methyl glucose.

The fate of 3-methyl glucose fed to the rat

The methods adopted followed closely those previously used by Cori (1926). The rats were arranged in pairs so that the total weight of each pair was approximately the same. The rats were starved and the urines collected for the 24 hr. period prior to the experiment. The rats in each group then received by stomach tube either 2·5 ml. of 50% (w/v) glucose or 3-methyl glucose, or 2·5 ml. water. The solutions were warmed to 37° before administration. The rats were immediately replaced in their metabolism cages in pairs and the urine collection started afresh. After a 4 hr. absorption period each rat was killed by an intraperitoneal injection of 0·5 ml. of Nembutal. As soon as the animal had lost consciousness the head was removed and as much blood collected as possible.

The liver was next removed, wiped free from blood on a filter paper, weighed and immediately placed in a boiling tube with 5 ml. of hot 60% (w/v) KOH. The bladder was

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emptied and the contents added to the sample of urine already obtained. The gut was ligatured at both ends, carefully dissected away, and placed in a beaker of distilled water. The body was completely eviscerated and skinned and the carcass placed in a beaker containing 60 ml. hot 60% KOH. Estimations were then carried out as follows. The liver and carcass glycogen content was determined according to the method of Evans, Tsai & Young (1931). The methoxyl content of 4 ml. samples of the acid hydrolysates of the precipitated glycogens was determined by the Zeisel method. The estimations of the latter were carried out in pairs, one estimation on a sample of an acid hydrolysate from a rat fed 3-methyl glucose and the other on a sample from a rat fed either glucose or water. Each pair of estimations was carried out on the same day with the same sample of HI.

As a check on the methoxyl content of the glycogens, some pure glycogen was isolated from the original alkaline hydrolysates of the carcasses in order to estimate the methoxyl content of the unhydrolysed glycogen samples. The method used for the isolation of the glycogen samples was that described by Somogyi (1934), their methoxyl content being estimated by the Zeisel method.

The extent of the absorption of the sugars from the intestine was determined by the method described by Cori (1926). The reducing power of the gut contents was estimated by the method of Hagedorn & Jensen. The amount of sugar fed to each rat was determined by passing an identical quantity of the sugar solution through the same catheter as that used in the rats into a volumetric flask, after which the volume was made up with distilled water.

Two 0·1 ml. samples of blood were taken from each rat and the blood sugar concentrations determined by the usual Hagedorn & Jensen technique. The rest of the blood was used to determine the concentration of 3-methyl glucose by the Zeisel method. The blood collected from each rat was added to a 25 ml. measuring cylinder containing 10 ml. 10% (w/v) trichloroacetic acid. After the blood had been well mixed with the acid the precipitate was removed on a dry filter paper and 4 ml. samples of filtrate were used for the methoxyl estimations. The filtrate from the blood of the glucose-fed rats served for a control estimation.

Benedict's qualitative test for reducing sugars was carried out on the urines from each of the three groups of rats. If sugar was present the amount was estimated by Benedict's quantitative method, and in the case of the 3-methyl glucose-fed rats the methoxyl content of the urines was also estimated by the Zeisel method. Since urinary sulphate interferes with the Zeisel estimation this had to be removed by treatment of the urine with hot 2N-HCl, followed by precipitation of the sulphate as $BaSO_4$. The filtrate was then used for the estimations. Control experiments showed that no significant amount of 3-methyl glucose was lost during this process.

The nature of the substances excreted after the administration of 3-methyl glucose and the recovery of the sugar in the urine

The volatile reducing substances in the urines were estimated by a method based on that described by Archer, Chapman, Rhoden & Warren (1948) for the determination of the volatile reducing substances in blood. Each determination was made on 2 ml. urine to which approx. 8 ml. water were added. Trouble due to frothing was overcome by the addition of a little glass wool to the distilling flask. The presence of glucose in the urine of rats fed 3-methyl glucose was tested by fermentation of a sample of the urine with baker's yeast at 28°.

Osazones were prepared from the urine of rats which had received 2 ml. of a 6% solution of 3-methyl glucose by intraperitoneal injection, and from the urine of normal rats to which a quantity of 3-methyl glucose had been added so that the methoxyl content of each urine was similar. Glucosazone was prepared from another urine to which sufficient glucose had been added to bring the concentration of sugar to approximately that of the experimental urines. The crystal structures of the osazones from the three groups of urines were compared under the microscope.

Two series of experiments were carried out in order to determine the extent to which the administered 3-methyl glucose could be recovered from the urine. In the first, the rats were given the sugar or water by mouth, as described above, and the urine collected by means of a separator as described by Maw (1948). The volume of urine from each group of rats was measured 4, 8 and 24 hr. after feeding, and the presence of reducing sugar tested with the Benedict quantitative reagent. The urine collection from the rats fed glucose and water was stopped after 24 hr., but the collection from the rats fed 3-methyl glucose was continued for several hours after the time at which the presence of reducing sugar could no longer be detected. Zeisel methoxyl estimations were then carried out on the urines. The rats were kept under observation for several weeks as it was of interest to know what long-term effect, if any, the treatment had.

In the second series of experiments the recovery of 3-methyl glucose was studied after an intraperitoneal injection of the sugar. Two pairs of rats, the total weight of each pair being approximately the same, were starved overnight. One pair was then given 2 ml. 3-methyl glucose (6%) solution and the other pair a similar quantity of 0·9% saline, by intraperitoneal injection. The urines were collected over the following 48 hr. during which time the rats were given a diet of cellulose. Urine was then collected from the experimental pair for a further 2 hr. and if this specimen failed to reduce Benedict reagent the collection of urine was stopped. The volume of urine excreted by each pair of rats during the 48 hr. period was recorded and the volume of the smaller sample brought to that of the larger by the addition of distilled water. The control urine was divided into halves. The same quantity of the same 3-methyl glucose solution as was given to one rat was then added to one half, and a similar quantity of 0·9% saline added to the other half. Zeisel methoxyl and Benedict estimations were then made on all the samples. These rats were also kept under observation for several weeks.

RESULTS

Glycogen content of liver and carcass

The livers of the rats given 3-methyl glucose by stomach tube possessed less than one-quarter, and those of the control rats given water less than one-ninth, of the glycogen content of those of the glucose-fed animals (Table 1). The differences between the mean results for the glucose-fed animals and

Table 1. *Glycogen content of livers and carcasses of rats given glucose or 3-methyl glucose by stomach tube*

(Results represent the mean for six animals. The glycogen content is expressed as equivalent glucose.)

Source of glycogen	Glycogen content (g./100 g. fresh tissue) of animals given (*a*) Glucose	(*b*) 3-Methyl glucose	(*c*) Water	Probability of difference being due to chance alone *a* and *b*	*a* and *c*	*b* and *c*
Liver	1·99±0·25	0·44±0·17	0·20±0·07	<0·001	<0·001	0·20–0·30
Carcass	0·26±0·031	0·12±0·012	0·11±0·008	0·001–0·01	<0·001	0·40–0·50

Table 2. *Methoxyl content of the glycogen from the liver and carcass of rats fed glucose and 3-methyl glucose, and of pure glycogen from each group*

Source of glycogen	'Apparent % OMe' in glycogen in animals given (*a*) Glucose or water	(*b*) 3-Methyl glucose	Probability of difference being due to chance alone *a* and *b*
Liver*	1·17±0·70	1·42±0·29	0·50–0·60
Carcass*	2·99±0·48	2·69±0·33	0·60–0·70
Pure glycogen from carcass	0·02 0·11	0·30	—

* Mean results for estimations on six different samples.

Table 3. *Concentration of sugar in the blood of rats given glucose or 3-methyl glucose by stomach tube*

Exp. no.	Concentration (mg./100 ml.) of sugar in the blood of animals given: Glucose Total reducing power (as glucose)	3-Methyl glucose: Total reducing power (as glucose)	3-Methyl glucose (from Zeisel)	Glucose (by difference)	Water Total reducing power (as glucose)
1	132	184	75	124	109
	130	176	80	112	104
2	134	246	187	123	100
	141	225	156	119	118
3	124	223	180	106	100
	135	232	189	110	93

those for the other groups are highly significant statistically, while the difference between the mean results for the rats given 3-methyl glucose and those given water is not statistically significant, on the number of animals in these experiments, at a 5% level of significance.

The carcasses of the rats given 3-methyl glucose and of those given water possess similar contents of glycogen (Table 1), amounting to about one-half of the glycogen content for the rats fed glucose. The mean figures for these two groups are not significantly different statistically. The differences between the mean figures for the glucose-fed rats and those for the other two groups are highly significant.

When acid hydrolysates of the glycogen from the livers and carcasses of the sugar-fed rats are examined for the presence of methoxyl groups (Table 2) no significant difference is found between the results for the animals given glucose and for those given 3-methyl glucose. The apparent presence of methoxyl groups in these crude hydrolysates is not to be attributed to the presence of methoxyl groups in the glycogen itself since the glycogen isolated from these tissues gave virtually negative methoxyl results (Table 2). The magnitude of these data for 'apparent % OMe' is to be attributed to the fact that blank experiments could not be carried out simultaneously when hydrolysates from glucose- or water-fed rats, and from 3-methyl glucose-fed rats were being simultaneously estimated. It was of course a comparison of the data for the two groups which was of importance, and not the absolute values.

Concentration of sugar in the blood of the sugar-fed animals

The blood-sugar level (calculated as glucose) of the 3-methyl glucose-fed rats is very much higher than the corresponding level for the rats fed either glucose or water (Table 3). Owing to the fact that the reducing power of 3-methyl glucose is less than that of glucose the real blood sugar level, as opposed to that referred to glucose, in the case of the 3-methyl glucose-fed rats is rather higher than that given in the table.

The concentration of 3-methyl glucose in the blood of the 3-methyl glucose-fed rats was determined from the Zeisel estimations and the approxi-

mate blood glucose concentration in these rats was calculated by difference. It will be seen that the concentration of glucose in the blood of the 3-methyl glucose-fed rats is substantially the same as that of the water-fed rats (Table 3).

Absorption of sugar from the gut

No significant amount of sugar was found in the gut of the glucose-fed rats, whereas in two experiments 100 and 180 mg. respectively of reducing sugar (calculated as 3-methyl glucose) were found in the filtrates of the gut contents of the 3-methyl glucose-fed rats. Since about 1 g. of sugar was fed to each rat, about 15% of the administered 3-methyl glucose was unabsorbed after the 4 hr. absorption period.

The nature of the substances excreted after the administration of 3-methyl glucose

The reduction tests on the urines excreted during the 4 hr. absorption period after feeding the rats by stomach tube showed that only the urines excreted by the 3-methyl glucose-fed rats reduced the Benedict qualitative reagent. Zeisel methoxyl and Benedict quantitative estimations were carried out on all these urines, using the urine excreted by the same rats during the previous 24 hr. as controls in the methoxyl estimations. Table 4 gives the concentration of sugar in the urines, assuming the latter to be 3-methyl glucose, as determined by the two methods, and the total amount of sugar excreted during the absorption period. The close agreement between the results obtained suggests that the sugar in the urine was probably 3-methyl glucose. Since about 2 g. of sugar were fed to the pair of rats about 25% of the fed sugar was excreted during the 4 hr. absorption period. It will also be observed that the concentration of the sugar in the urine is about 6%, which is approximately the strength of a solution of a hexose isotonic with the blood.

Table 4. *Estimation of 3-methyl glucose in the urine of rats given 3-methyl glucose by stomach tube*

Exp. no.	Concn. of 3-methyl glucose (mg./ml.) in the urine as estimated by: Benedict's method	Zeisel's methoxyl method	Total amount of 3-methyl glucose excreted during absorption period (Zeisel's method) (mg.)
1	65	71	750
2	46	47	550
3	68	62	550

Table 5 shows the titration readings obtained in the volatile reducing matter estimations with the urine of rats which had been fed 3-methyl glucose, glucose and water. There is no significant difference between the results obtained from the three groups and it is concluded, therefore, that there is no significant increase in the volatile reducing matter, e.g. methanol or formic acid, in the urine as a result of feeding 3-methyl glucose to a rat.

Table 5. *Estimation of volatile reducing matter in the urine of rats given glucose or 3-methyl glucose by stomach tube*

Urine sample	Concn. (ml. 0·1 N-$Na_2S_2O_3$/ml. of urine) of volatile reducing matter in the urine of rats given: Glucose	3-Methyl glucose	Water
24 hr. before feeding	0·84, 0·76	0·86, 0·81	0·86, 0·80
24 hr. after feeding	0·84, 0·80	0·82, 0·74, 0·79	0·74, 0·71

Fermentation studies on the urines showed that there was no abnormal amount of glucose present as a result of feeding 3-methyl glucose.

There was an obvious similarity in the crystal structure of the osazones prepared from the urines excreted by the rats which had been administered 3-methyl glucose and the urine to which 3-methyl glucose had been added. These crystals differed markedly from those of the osazones prepared from urine to which glucose had been added. Thus it is concluded that the urines from the rats which had received an intraperitoneal injection of 3-methyl glucose contained a considerable amount of the sugar.

Recovery of sugar in the urine

In the recovery experiments the urine excreted by the rats fed 3-methyl glucose reduced the Benedict qualitative reagent 4, 8, 24 and 28 hr. after feeding by stomach tube, but urine collected at 31 hr. failed to reduce the reagent. The amount of 3-methyl glucose excreted in the urine up to that time was determined. In two experiments recoveries of 71 and 80% were obtained. None of the rats used in these experiments showed any pathological symptoms while under observation.

Table 6. *Recovery of 3-methyl glucose in the urine of rats given 3-methyl glucose (6% solution) by intraperitoneal injection*

Exp. no.	Comparison of Zeisel methoxyl titres (ml. 0·01 N-$Na_2S_2O_3$/total urine volume) for the urines containing: Excreted 3-methyl glucose	Added 3-methyl glucose	Recovery of the injected sugar in the urine (%)
1	10·3	11·9	87
2	7·90	8·13	97

In the experiments in which 3-methyl glucose was given by intraperitoneal injection, no urine collected more than 48 hr. after the injection of the sugar reduced the Benedict qualitative reagent. The methoxyl determinations were, therefore, carried out on the urines excreted during this 48 hr. period. In this case a comparison of the titration readings from the Zeisel estimation for the control urine to which 3-methyl glucose had been added and the urine excreted by the 3-methyl glucose-fed rats provides the best measure of the recovery of the administered sugar. The results of the two experiments are summarized in Table 6. The average recovery in the two experiments was 92 %. Thus the recovery was rather higher when the sugar was administered by intraperitoneal injection than when it was fed by stomach tube. The reducing power of the excreted sugar was identical with 3-methyl glucose. Again no pathological symptoms developed in any of the rats kept under observation after the completion of the experiments.

The diuretic effect of 3-methyl glucose

The volume of urine excreted by the three groups of rats during the 4 hr. absorption period after feeding glucose, 3-methyl glucose and water by stomach tube was measured. Approximately twice as much urine was excreted by the six 3-methyl glucose-fed rats (30·9 ml.) during the period as by either of the other two groups of rats (12·0 and 14·7 ml.). In Table 7 the urine volumes in a similar experiment which was continued for a rather longer period are recorded. This shows that while 3-methyl glucose has a diuretic effect for about the first 8 hr. after feeding, over a 24 hr. period no diuretic effect is observable.

Table 7. *Volume of urine excreted by rats given glucose and 3-methyl glucose by stomach tube*

Period of excretion of urine	Volume of urine (ml.) excreted by pairs of animals given: Glucose	3-Methyl glucose	Water
24 hr. before feeding (av. for Exps. 1 and 2)	17	20	18
7 hr. after feeding (Exp. 2)	10	15	9·2
8 hr. after feeding (Exp. 1)	6·2	19	6·4
24 hr. after feeding (av. for Exps. 1 and 2)	17	20	18

DISCUSSION

The methoxyl determinations on the acid hydrolysates of the glycogen samples from the carcass and liver after feeding 3-methyl glucose showed that no significant amount of methyl glycogen is formed in the liver. A comparison between the carcass glycogen levels of the rats fed 3-methyl glucose, glucose and water suggests that 3-methyl glucose does not give rise to any significant amount of carcass glycogen. Although there is statistically no difference between the liver glycogen contents in the water- and 3-methyl glucose-fed rats the mean content for the latter group was twice that for the former. The possibility that 3-methyl glucose can be utilized to a very small extent cannot be excluded. Since Hurlock & Tosic (1951) have found a strain of micro-organism that will utilize 3-methyl glucose, it is perhaps not surprising if some small degree of utilization of methyl glucose in the animal body does indeed occur.

The observed rise in the concentration of sugar in the blood after feeding 3-methyl glucose would be expected with a sugar which is not being readily metabolized. The fact that the glucose concentration of the blood is not seriously affected suggests that 3-methyl glucose has little or no effect on the hormonal control of the blood-sugar level.

Our results concerning the rate of absorption of 3-methyl glucose from the gut are in general agreement with the view that this sugar is actively absorbed (Campbell & Davson, 1948; see also Csáky, 1942).

The experiments on the nature of the substances excreted after the administration of 3-methyl glucose show that the unchanged sugar is present in the urine. The fact that there was no significant increase in the concentration of the volatile reducing matter in the urine after feeding the sugar, suggests that there is no extensive breakdown of the sugar, for this would probably lead to the formation of either methanol or formic acid, either of which substances would have affected the content of volatile reducing substances in the urine.

A rather higher rate of recovery of the sugar in the urine might be expected when the sugar is fed by intraperitoneal injection, rather than by stomach tube, as in the former case the possibly complicating factor of intestinal absorption is absent. The average figure for the recovery from the urine (92 %) is rather low if indeed the sugar is not metabolized at all, although this low recovery may result from the experimental difficulties involved. Nevertheless, the possibility that a very small amount of the sugar is indeed metabolized cannot be excluded.

SUMMARY

1. The administration of a solution of 3-methyl glucose to normal rats did not lead to a significant increase in the glycogen content of the liver or carcass nor to the formation of any significant amount of monomethyl glycogen in the liver or carcass.

2. No significant increase in the amount of volatile reducing substances in the urine took place after the administration of 3-methyl glucose to the rat, but a reducing sugar shown to be unchanged 3-methyl glucose was excreted.

3. An average recovery in the urine of 92 % of the administered sugar was obtained after an intraperitoneal injection of 3-methyl glucose to the rat.

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Metabolic Studies with 3-Methyl Glucose

2. *IN VITRO* STUDIES

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From the experiments reported in the previous paper (Campbell & Young, 1952) it appears improbable that 3-methyl glucose is metabolized to any appreciable extent *in vivo* in the rat. It was, therefore, of interest to determine whether any evidence for its metabolism could be obtained from *in vitro* studies. First, the effect of 3-methyl glucose on the rate of fermentation of glucose by yeast was studied. Secondly, since Cori, Cori & Green (1943) had shown that glucose strongly inhibited the conversion of glucose-1-phosphate to polysaccharide with a crystalline muscle phosphorylase preparation it was of interest to know if 3-methyl glucose possessed similar properties. Finally, an attempt was made to synthesize 3-methyl glucose-1-phosphoric acid so that the suitability of this substance as a substrate for phosphorylase could be studied.

EXPERIMENTAL

Experiments with yeast

The effect of 3-methyl glucose on the rate of fermentation of glucose by yeast was studied by following the loss of reducing power of a reaction mixture with and without the addition of 3-methyl glucose during the fermentation of glucose by yeast. It was first necessary to determine the conditions under which the loss of reducing sugar was linearly related to time. The fermentations were carried out in a similar manner to that already described for the estimation of 3-methyl glucose in plasma (Campbell & Young, 1952), using 2 ml. of yeast suspension, 2 ml. of 5 % (w/v) glucose solution, and 2 ml. of 5 % 3-methyl glucose solution or water. The fermentation was stopped at 0, 10, 20 and 30 min. respectively by the addition of 2 ml. of a 5 % solution of $ZnSO_4$ and 2 ml. of saturated $Ba(OH)_2$ solution, quickly followed by removal of the yeast by filtration. All fermentations were carried out at the same time so that the conditions were as nearly as possible the same in all cases. The reducing power of a 3-methyl glucose solution before and after incubation with yeast was determined as a control experiment.

Experiments with a muscle phosphorylase preparation

The experimental procedure was based on that of Cori *et al.* (1943) in which the synthesis of a polysaccharide from glucose-1-phosphate with crystalline muscle phosphorylase is followed by measuring the rate of increase of inorganic phosphate in the reaction mixture.

Preparation of reactants. Crystalline muscle phosphorylase was prepared from rabbit muscle by the method of Green & Cori (1943) but differed from that procedure in two respects. First, the animal was killed by the $MgSO_4$-

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