Sugar Transport by the Bacterial Phosphotransferase System

THE GLUCOSE RECEPTORS OF THE SALMONELLA TYPHIMURIUM PHOSPHOTRANSFERASE SYSTEM*

(Received for publication, February 5, 1982)

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We have previously reported that glucose can be phosphorylated by phospho-HPr and two sugar-specific pairs of proteins of the Escherichia coli and Salmonella typhimurium phosphoenolpyruvate:glycose phosphotransferase system. Each of the sugar-specific complexes comprises two proteins, lipid, and divalent cation, and each is present in membranes isolated from wild type cells. For reasons described in this report, one of the complexes is designated II^Glc and the other II^Man. The II^Man complex has previously been separated into its protein components, II-A and II-B (Kundig, W., and Roseman, S. (1971) J. Biol. Chem. 246, 1407-1418), while the accompanying reports describe dissociation of the II^Glc complex into its components, III^Glc and II-B^Glc.

Curtis and Epstein (Curtis, S. J., and Epstein, W. (1975) J. Bacteriol. 122, 1189-1199) first showed that there are two phosphotransferase systems in whole cells responsible for glucose uptake and obtained the respective mutants, now designated ptsG and ptsM. The present studies provide kinetic conditions for assaying each activity separately (in vivo and in vitro), when both are present in the same membrane preparation. The II^Glc system is responsible for the uptake and phosphorylation of glucose and methyl a-glucoside, whereas the II^Man system is less specific and utilizes glucose, mannose, and 2-deoxyglucose. With high sugar concentrations in vitro, II^Man is also capable of phosphorylating methyl a-glucoside, fructose, and N-acetylmannosamine, while II^Glc phosphorylates fructose and mannose.

The in vivo transport results were qualitatively consistent with the in vitro phosphorylation results, and several of the kinetic parameters also showed good quantitative agreement.

The levels of the two activities depended on the growth conditions. In addition, transport studies showed that initial uptake rates of methyl a-glucoside and steady state levels of this analogue depended on the energy state of the cells and that these two parameters did not necessarily change in the same direction when metabolic inhibitors were used.

A series of E. coli and S. typhimurium mutants were characterized both with respect to their ability to transport the glucose analogues and to phosphorylate them in vitro. The original mutants of Curtis and Epstein, ptsG and ptsM, were found to be defective in II-B^Glc and the II^Man complex, respectively.

The accompanying papers (1-3) describe the isolation and properties of two general proteins of the Salmonella typhimurium phosphotransferase system, Enzyme I and HPr, and of a glucose-specific protein, III^Glc. In enteric bacteria, such as S. typhimurium and E. coli, glucose can be taken up via four known transport systems, two mediated by the PTS, and two by non-PTS galactose permeases. The fact that glucose can enter the cell via the galactose permeases, and then be phosphorylated by glucokinase plus ATP, explains why mutants defective in one of the PTS general proteins, such as Enzyme I, can grow on glucose, provided that the cells have been induced for one (4) or other of the galactose permeases (5).

A number of studies have been reported in which mutants defective in one or more of the glucose-specific proteins of the PTS were used. With these mutants, it was shown that the PTS is involved in chemotaxis of the organism toward its substrates (6) and in regulating adenylate cyclase (7). Curtis and Epstein (8) described mutants of this type and showed (a) that two different Enzyme II systems could utilize glucose, (b) that mutants defective in one or both of these complexes could be obtained, and (c) that one system, hereafter designated II^Glc, showed a narrow specificity (for glucose and methyl a-glucoside), while the other, II^Man, exhibited a broad specificity (for glucose, mannose, 2-deoxyglucose, glucosamine, and mannosamine). What was not clear from these studies was how the II^Glc and II^Man complexes were related to the previously described II-A/II-B and III^Glc/II-B^Glc (1-3) complexes, i.e. which complex was defective in a given mutant.

The abbreviations used are: HPr, histidine-containing phospho-carrier protein of the phosphotransferase system; PTS, phosphoenolpyruvate:glycose phosphotransferase system; P-enolpyruvate, phosphoenolpyruvate. The designations of the other components of the system are given in Fig. 1 of the first accompanying paper in this series (1). Unless otherwise stated, all sugars are of the p configuration and glycosides are pyranosides.

The nomenclature used here corresponds to the following genetic designations employed by Curtis and Epstein (8): II^Man is the product of the ptsM gene(s); II-B^Glc is the product of the ptsG gene(s).
The present report describes the kinetic and specificity properties of the two glucose complexes, both \textit{in vitro} and \textit{in vivo}. To avoid the problems encountered in using metabolizable sugars in transport experiments, the \textit{in vivo} experiments were conducted with the analogues, methyl \(\alpha\)-glucoside and 2-deoxyglucose. 

**Experimental Procedures**

\textbf{Transport Measurements—} Unless otherwise stated, the following protocol was used to measure sugar transport: a 1.0-ml aliquot of cells (see above) was added to 1.0 ml of modified Medium 63. The resulting suspension was warmed to 25 °C, and all subsequent operations were conducted at this temperature. After warming, the 2 ml of cells were rapidly mixed with 2 ml of modified Medium 63 containing \(^{14}\)C-sugar using the stopped flow apparatus depicted in Fig. 1. Mixing was complete within 1 s. Aliquots (0.1 ml) were removed at 3- to 5-s intervals with an autopipette connected to a Heathkit BX recorder (Fig. 1) and immediately dispensed on modified Medium 63, which was then passed through a Whatman GF/F glass fiber filter (2.4-cm diameter) mounted on a Millipore 3025 sampling manifold. The total time between dilution of each 0.1-ml aliquot and its complete filtration was less than 3 s. The initial mixing of cells with \(^{14}\)C-sugar and each operation of the autopipette were timed on the recorder. The time for each uptake determination was taken as the interval between mixing of cells with sugar (time zero) and dilution into 10 ml of medium. Control experiments showed that the uptake of sugars was essentially stopped by the dilution step, that the filters quantitatively retained the bacteria, and that the extent of efflux from cells in the dilution medium was insignificant. The amount of \(^{14}\)C-sugar accumulated as a function of time could thus be determined from the \(^{14}\)C-sugar retained on the filters. This was assayed in a Packard liquid scintillation spectrometer. The values obtained were corrected for extracellular \(^{14}\)C-sugar adsorbed to filters estimated by following the protocol in the absence of cells. With the use of labeled solutes that were not actively accumulated, it was shown that this control provided an accurate measure for background levels of \(^{14}\)C-sugar adsorbed to filters in the presence of cells (19). For all substrates used, the background was equivalent to the amount of label bound to the filters in the original labeled suspension. The rate of sugar uptake was estimated by inspection of progress curves of sugar accumulation derived from not less than seven measurements of intracellular \(^{14}\)C-sugar taken over a 30-s interval.

**Phosphorylation in Vivo**—Phosphorylation of \(^{14}\)C-sugars by intact cells was routinely measured in the same way as transport except that the 0.1-ml samples were not diluted but were dispersed into small tubes containing 0.3 ml of ethanol at \(-80^\circ\) C. The resulting 0.4-ml samples were later heated for 5 min in a 75 °C water bath, and transferred to small columns (1 x 6 cm) of AG 1-X2, 50-100 mesh, ion exchange resin in the Cl form which had been exhaustively rinsed with water. Sugar phosphate accumulation by intact cells was also measured as follows: samples were diluted and filtered as in a routine transport assay. A powder of solid CO\(_2\) was then placed on top of each filter. The filters plus frozen cells were later extracted into 3 ml of ethanol at 70 °C and the extracted material was transferred to the ion exchange columns as described above. In each procedure, two 10-ml water washes were used to remove undervatilated neutral sugar from the ion exchange column, and sugar phosphate was eluted by two 3-ml washes with 1 M LiCl. Eluent was collected in liquid scintillation vials to which 6 ml of scintillation fluid were added, and radioactivity was assayed in a Packard liquid scintillation spectrometer. This method for sugar phosphate analysis is described in an accompanying report (1). Analysis of sugar phosphate in filtered cells and in total cell suspensions gave the same values when the two were performed simultaneously.

**Assays of Phosphotransfer Proteins**—The assay procedures used in this study are described in the accompanying papers (1-3). The assay of hexokinase refers to the coupled assay where the production of pyruvate reflects the rate of utilization of phosphoenolpyruvate by Enzyme I (20).

**Sugar Phosphorylation in Vivo**—The Enzyme II complexes were assayed as described elsewhere (20) with Enzyme I in large excess. The specific activity of \(^{14}\)C-sugar was maintained constant at all concentrations in each individual experiment. Typical incubation mixtures contained the following components in final volumes of 0.1 ml: 0.05 M potassium phosphate, pH 7.5; 2.0 mM dithioerythritol; 12.5 mM KF; 5 mM MgCl\(_2\); 10 mM phosphoenolpyruvate; 10 units of Enzyme I; and sugar substrate as indicated (6-10 times the respective \(K_c\) value for quantitating a given Enzyme II). In mannose receptor (II\(^{MSS}\)) assays, HPr was used at 25 μM concentrations. Glucose receptor (II-H\(^{B2}\)) was assayed using very low concentrations of HPr (see "Results") and II\(^{4}\) at about 4 μM concentrations (see "Results"). The accompanying papers (1-3) describe the methods used for preparation of Enzyme I, HPr, and II\(^{4}\), as well as the methods used for preparing soluble and membrane fractions. For routine assays, highly purified or homogeneous HPr was used, Enzyme I was 30- to 100-fold purified, and II\(^{4}\) was purified at least 30-fold.

At each sugar concentration, measurements were made at 10, 20, and 30 min to ensure that rates of phosphorylation were constant with time of incubation. Sugar phosphorylation never exceeded 10% of the total sugar added to incubation mixtures. The effects of substrate concentration on initial rates were plotted by following the standard methods: \(v\) versus \(S\); \(v/\varepsilon\) versus \(S/\varepsilon\); \(v/\varepsilon\) versus \(S\), where \(v\) is initial velocity and \(S\) is the substrate concentration (21). In all instances, the estimations of \(V_{max}\) and \(K_c\) from these treatments of the data showed good agreement.

It is important to emphasize that the mannose receptor, II\(^{MSS}\), consists of a protein complex, the II-A/II-B\(^{MSS}\) system (22). No attempt was made to assay the individual proteins of this complex, since although they are separable (22), the separation is not quantitative and results in substantial losses in total activity when the reconstituted complex is compared with the activity of the original membrane preparation.

**Results**

**Glucose Transport in \textit{S. typhimurium}**—A number of analogues of glucose have been used to study glucose transport in \textit{E. coli} and \textit{S. typhimurium}. Methyl \(\alpha\)-glucoside has been used extensively by many workers in part because it is not metabolized beyond its phosphorylation (23), while the transport of 2-deoxyglucose (which is also a mannose analogue) has been studied to a much lesser degree. Curtis and Epstein (8) have shown that two glucose transport systems can be distinguished in \textit{E. coli} by their ability to transport either methyl \(\alpha\)-glucoside or 2-deoxyglucose and by the ability of other hexoses to inhibit the transport of each analogue. Thus, we decided to study further the kinetcs of the uptake and phosphorylation of the analogues \textit{in vitro} on aliquots of the same cells that were used for studying the kinetic properties of the Enzymes II \textit{in vitro}, so that these could be compared.

We first examined the uptake of 2-deoxyglucose. The results presented in Fig. 2 (Miniprint) confirm that 2-deoxyglucose is a PTS transport substrate. At the earliest time points, all of the sugar is accumulated as its phosphate ester. Subsequently, the free sugar appears, apparently due to the action of intracellular phosphatases. Methyl \(\alpha\)-glucoside is also accumulated as the phosphate ester and, over a longer time course (not shown in Fig. 2), is also partially hydrolyzed intracellularly. Strain SB2309, which has a deletion in \(ptsL\) and \(ptsH\), cannot accumulate either analogue.

The kinetic values for uptake of 2-deoxyglucose and methyl \(\alpha\)-glucoside are shown in Tables II and III, respectively. The II\(^{4}\) system is responsible for the uptake of 2-deoxyglucose...
and is a high capacity system as shown by the kinetic results in Table II. In addition, the inhibition studies indicate that glucose and mannose are effective substrates of the IIM\textsuperscript{Mφ} complex, while fructose is not an inhibitor, at least of 2-deoxyglucose uptake. These results correlated well with the \textit{in vitro} data given below. Finally, as indicated in Table II, the analogue used for the original studies with the PTS (24), N-acetyl-D-mannosamine, proved to be a poor substrate of IIIM\textsuperscript{Mφ}.

The results with methyl α-glucoside, shown in Table III, clearly indicate that the II\textsuperscript{Mφ} complex is much more specific than the IIIM\textsuperscript{Mφ} complex. The only effective inhibitors of methyl α-glucoside uptake were glucose, as shown in the table, and methyl β-glucoside, as first noted by Hoffman et al. (25) (data not shown).

\textbf{In Vitro Phosphorylation Experiments—}A complete characterization of Enzymes II requires sugar specificities and kinetic constants for the different sugars. Such studies are planned after the homogeneous proteins have been isolated. However, we have already shown that reconstitution of purified II-B\textsuperscript{Mφ} activity requires phosphatidylglycerol and divalent cation to be added in a specific order (22). This suggests that the kinetic characteristics of the reconstituted preparations may not be the same as those found with the intact cells or washed membrane preparations, which contain membrane proteins subjected to only minimal perturbation. For this reason, \textit{in vitro} phosphorylation experiments were conducted with intact, washed membranes.

Such preparations from wild type cells contain the following integral proteins: the IIIM\textsuperscript{Mφ} system (II-A\textsuperscript{Mφ}/II-B\textsuperscript{Mφ}/II-C\textsuperscript{Mφ}), and variable small quantities of the peripheral proteins Enzyme I, HPr, and III\textsuperscript{Mφ}. The peripheral proteins are often present only in trace quantities, but these cannot be removed by repeated washing in high and low ionic strength buffers.

Measurement of either IIIM\textsuperscript{Mφ} or II-B\textsuperscript{Mφ} in crude membrane preparations depends on the relative quantities of HPr and III\textsuperscript{Mφ} used in the assays, and we can utilize these differences to measure one enzyme complex in the presence of the other. For routine assays, we used the following incubation conditions: (a) II-B\textsuperscript{Mφ}, excess Enzyme I, 4 \textmu M III\textsuperscript{Gφ}, but no HPr other than endogenous protein bound to the membranes; (b) IIIM\textsuperscript{Mφ} complex: the same conditions except that 20 \textmu M HPr was included and III\textsuperscript{Gφ} was omitted. The reasons for these conditions are given below.

With II-B\textsuperscript{Gφ}, the apparent \(K_m\) measured for III\textsuperscript{Gφ} is 3 to 4 \textmu M (3). Thus, we routinely added III\textsuperscript{Gφ} to a concentration of at least 4 \textmu M to measure II-B\textsuperscript{Mφ} activity.\footnote{N-Acetyl-D-mannosamine was also tested but was not an efficient substrate of the IIIM\textsuperscript{Mφ} system; the rate of uptake by wild type \textit{S. typhimurium} cells was about 6.5% of the rate observed with 2-deoxyglucose, and phosphorylation \textit{in vitro} was reduced to about 16%. Uptake was strongly inhibited by glucose, mannose, and even by fructose.} We also measured the \(K_m\) for HPr in this system and found it to be extremely low and that endogenous HPr or traces of exogenous HPr were sufficient to give optimal rates of methyl α-glucoside phosphorylation. Thus, in the presence of trace quantities of HPr, we were able to measure II-B\textsuperscript{Mφ} activity. This is in marked contrast to the IIIM\textsuperscript{Mφ} system. Here, measurement of the \(K_m\) for HPr gave values of about 20 \textmu M, and therefore, we routinely used 25 \textmu M HPr in our assays. Under these conditions, the amount of endogenous III\textsuperscript{Gφ} present in the membranes was insufficient to permit expression of II-B\textsuperscript{Mφ} activity, so that we were able to measure activity of the IIIM\textsuperscript{Mφ} system.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Hexose} & \textbf{Transport } & \textbf{Phosphorylation } \\
& \textbf{KS} & \textbf{Vmax} & \textbf{KS} & \textbf{Vmax } \\
\hline
Glucose & 0.02 & 0.01 & 126 & 0.04 \textsuperscript{a} \\
Mannose & 0.04 & & & \\
Fructose & 0.02 & & & \\
2-Deoxyglucose & & & & \\
\hline
\end{tabular}
\caption{Kinetics of Enzyme II-B\textsuperscript{Gφ} activity toward various hexoses}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Sugars} & \textbf{Transport} & \textbf{Phosphorylation} \\
& \textbf{KS} & \textbf{Vmax} & \textbf{KS} & \textbf{Vmax} \\
\hline
2-Deoxyglucose & 0.18 & 400 & 0.20 & 72 \\
Glucose & 0.02 & 0.015 & 44 & 110 \\
Mannose & 0.02 & 0.045 & 44 & 110 \\
Fructose & 0.02 & 20.0 & 72 \\
Methyl α-glucoside & 25.0 & 92 \\
\hline
\end{tabular}
\caption{Kinetics of Enzyme II\textsuperscript{Mφ} activity toward various hexoses}
\end{table}
without interference by II-BMa". It is possible, therefore, to measure either of the glucose receptor activities by utilizing the appropriate quantity of HPr or III'Gc in the assay system.

Kinetic experiments in vitro were conducted with different membrane fragments of II-Mac and/or III'Gc systems. In addition, membranes were obtained from cells grown on different carbon sources. Fig. 3 (Miniprint) shows that methyl α-glucoside can be phosphorylated by the II-Mac system (in the absence of III'Gc), with an apparent $K_m$ for the sugar of about 25 mM, regardless of whether lactate or glucose was the carbon source used for growth of the cells, and with membranes from both wild type cells and the deletion mutant. The apparent $K_m$ of 25 mM agrees with our previously reported value of 20 mM which was obtained before discovery of III'Gc and the II-Mac system. Fig. 3 also shows that, when III'Gc is added to the assay mixtures, the high affinity II-Mac system for methyl α-glucoside phosphorylation becomes active.

In addition to varying the amounts of HPr and III'Gc, the two systems can be identified by inhibition with other sugars (data not shown). At high concentrations of HPr (30 μM) and no added III'Gc, the phosphorylation of glucose (10 μM) by the II-Mac system was strongly inhibited by mannose and 2-deoxyglucose, but less than 10% inhibited by 1 mM concentrations of methyl α- and β-glucoside. By contrast, when the assays with the same membranes and inhibitors were performed in the presence of 2 μM HPr and 4 μM III'Gc, methyl α- and β-glucosides were effective inhibitors, while mannose and 2-deoxyglucose were relatively ineffective.

Comparison of Glucose Receptor Activities in Vivo and in Vitro—The kinetic parameters for transport in vivo and phosphorylation in vitro are given in Tables II and III. (In the discussion that follows, all kinetic parameters are apparent values.) In view of the complexity of the kinetic equations which describe the PTS (9), which differ in vitro and in vivo, we did not necessarily expect good agreement in kinetic constants. Surprisingly, however, the $K_m$ values in vitro and in vivo shown in Table II for the II-Mac system with 2-deoxyglucose show good agreement, suggesting that we had fortuitously chosen appropriate conditions for the measurement of these values. For example, the II-Mac system exhibited excellent phosphorylating activity in vitro with three sugars. The $K_m$ values were: glucose, 15 μM; mannose, 45 μM; and 2-deoxyglucose, 200 μM. The other two sugars, fructose and methyl α-glucoside, were poor substrates, with $K_m$ values of 20–25 mM. Essentially the same results were obtained in the transport experiments; 2-deoxyglucose was an effective substrate of the transport system with a $K_m$ of 180 μM and transport was strongly inhibited by glucose ($K_m$ 20 μM) and mannose ($K_m$ 20 μM), whereas no inhibition was detected with fructose. Methyl α-glucoside was not tested. Furthermore, the in vitro $K_m$ and in vivo $K_v$ values for the different sugars showed surprisingly good agreement.

As shown in Table II, the $V_{max}$ value for uptake of 2-deoxyglucose (400 μmol/g of cells, dry weight/min) differs from the value for in vitro phosphorylation (72 μmol/g/min). While the latter value can be increased at least 2-fold by increasing the HPr concentration in the in vitro assay to 100 μM (approximately physiological concentrations, the in vitro phosphorylation rates are much below the in vivo transport rate, especially since the in vitro studies were conducted at 37 °C, while transport was measured at 23 °C. Attempts to increase the in vitro activity of the II-Mac system were unsuccessful; these included treatment of the membranes with tolueno, phosphatidylglycerol (22), Triton X-100, sonic disruption, and passage through a French press. It is possible that the membranes cause dissociation of II-A"Mac and II-B"Mac to different membrane fragments.

Table III compares the in vitro and in vivo results for the II-Gc system. Of the five sugars tested, only glucose and methyl α-glucoside are effective substrates of this system. The $K_m$ value for glucose phosphorylation in vitro (10 μM) agreed well with its $K_m$ value (20 μM) as an inhibitor of methyl α-glucoside transport. The $K_m$ values for methyl α-glucoside, however, 6 μM for phosphorylation and 170 μM for transport, were not in good agreement. Fructose, mannose, and 2-deoxyglucose are poor substrates of this system, although some inhibition of methyl α-glucoside uptake was found when high concentrations of fructose and mannose were used. The slight in vitro phosphorylating activities with these sugars may be due to the II-Mac system present in the membranes rather than to the III'Gc system. From all of these results, it appears that the III'Gc system is specific for methyl α-glucoside and glucose below concentrations of 1 mM.

The $V_{max}$ values for the II-Gc in vitro phosphorylating and in vivo transport activities were measured at 37 and 25 °C, respectively. When corrected for the temperature differences, the in vitro phosphorylating activity is less than, but reasonably close to, the in vivo uptake rate, 50 versus 77 μmol/g of cells, dry weight, per min, respectively. Thus, the in vitro and in vivo $V_{max}$ values agree, but the $K_m$ values do not. We have no explanation for these results at this time.

Regulation of PTS Activities: Effect of Growth Medium—We have previously reported (27) that the levels of Enzyme I and HPr in S. typhimurium can vary about 3-fold depending on the carbon source used for the growth of the cells. We used the assays described above to determine how the carbon source used for growth affected the II-Gc and II-Mac systems; the results are shown in Table IV.

In agreement with previous results (27), growth of wild type cells on glucose or fructose yielded about a 3-fold increase in activity of Enzyme I and HPr in crude extracts compared to cells grown on lactate. The III'Gc activity also increased, but no more than 2-fold, and essentially the same results were obtained by assaying membranes for II-Mac. (Either II-A"Mac or II-B"Mac could be induced or derepressed to much higher levels, but the assay would measure only the rate-limiting protein.) If the II-A"Mac/II-B"Mac complex is entirely responsible for the uptake of 2-deoxyglucose, and the III'Gc/II-B"Mac complex for methyl α-glucoside uptake, and further, if these activities are rate-limiting and are not regulated intracellularly, then one would make the following predictions based on the activities shown in Table IV. Cells grown on mannose, fructose, and glucose should take up 2-deoxyglucose at twice the rate of cells grown on lactate or glycero. Similarly, methyl α-glucoside uptake should also increase. Mannose-grown cells generally fit this pattern, i.e. the $V_{max}$ of uptake of both analogues was approximately 2-fold greater for mannose-grown cells than for lactate- or glycero-grown cells. Glucose-grown cells also fit the general pattern with respect to methyl α-glucoside uptake, the increase over lactate- and glycero-grown cells being about 3-fold. The uptake of 2-deoxyglucose by glucose-grown cells was somewhat higher than expected from the foregoing discussion, but this value was again only 3-fold over lactate-grown cells.

By contrast, anomalous results were obtained by growth of the cells on fructose. These cells contained increased quantities of PTS proteins. However, instead of an increased rate of uptake of the glucose analogues, fructose-grown cells showed a substantial decrease, especially in their ability to take up 2-

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* Methyl β-glucoside is probably also an effective substrate, but since there is a known β-glucoside PTS transport and phosphorylating system which could interfere with the present measurements (26), no extensive studies were conducted with this sugar in the present experiments.
deoxyglucose relative to cells grown on lactate and glycerol. We have previously reported (20) that growth of the cells on fructose results in induction of a protein activity that resembles, but is clearly distinct from, HPr. Whether this activity down-regulates the PTS when glucose analogues are used for transport remains to be determined.

Since many bacterial catabolic systems are induced by nonmetabolizable substrates (i.e. gratuitous inducers), cells were grown in the presence of 2-deoxyglucose and methyl α-glucoside to determine whether these substrates might induce the relevant PTS proteins. We are aware that these glucose analogues are accumulated as the corresponding phospho derivatives by the cells and that sugar phosphates are known to be toxic when accumulated at high levels by bacteria. Nevertheless, it was still possible that these substances acted as efficient inducers. The results are shown in Table V and were quite the reverse of those expected. The cells were not induced for PTS activities but were, in fact, strongly repressed in their synthesis of some of the PTS proteins.

It was surprising to find that only the sugar-specific proteins were affected by the analogues. The levels of Enzyme I, HPr, and other proteins tested were not significantly decreased, indicating that 2-deoxyglucose and methyl α-glucoside did not have a generally deleterious effect on protein synthesis. Of the sugar-specific proteins, IIIa was decreased 2- to 4-fold, IIMan 3- to 5-fold, and II-BGlc 3- to 6-fold. It was especially interesting that each analogue affected both systems; IIMan, for example, was almost as sensitive to growth in the presence of methyl α-glucoside as it was to 2-deoxyglucose. These data suggest the possibility that a common element may be regulating the synthesis of these membrane proteins, or perhaps a common polypeptide chain is involved in their function.

The transport studies confirmed the in vitro phosphorylation assays. Growth of the cells in the presence of the analogues resulted in a marked decrease in the ability of such cells to take up the analogues. 2-Deoxyglucose uptake was particularly sensitive, being decreased about 20-fold; the decrease in methyl α-glucoside uptake (Vmax) ranged from about 3- to 9-fold.

The results in Tables IV and V, therefore, show that,

| Table IV |

Phosphotransferase activities in cells grown on various carbon sources

*S. typhimurium* SB3507 was grown on the indicated carbon source, harvested, and prepared for either in vitro assays or transport determinations as described under "Experimental Procedures." Activity assays were performed as outlined in Tables II and III with the use of 1 mM methyl α-glucoside and 10 mM 2-deoxyglucose. Results are presented relative to those obtained in glucose-grown cells. These values for II-BGlc and IIMan are given in Table II and III. The values for the other PTS proteins in crude extracts of glucose-grown cells were: Enzyme I, 24.6 μmol of sugar phosphorylated/mg of protein/30 min when assayed as described (20); HPr, 0.77, and IIIaGlc, 0.46 mmol/ mg of protein (20).

| PTS activity | Glucose | Mannose | Fructose | Glycerol | D-Lactate |
|---------------|---------|---------|----------|----------|----------|
| *In vitro* activities |
| Enzyme I      | 1.00    | 0.68    | 0.84     | ND*      | 0.36     |
| HPr           | 1.00    | 0.69    | 0.86     | 0.36     | 0.34     |
| IIIaGlc       | 1.00    | 0.57    | 0.78     | 0.63     | 0.46     |
| II-BGlc       | 1.00    | 0.40    | 0.49     | ND*      | 0.09     |
| IIMan         | 1.00    | 0.89    | 1.26     | 0.63     | 0.52     |
| Vmax of transport |
| Methyl α-glucoside | 1.00 | 0.53    | 0.21     | 0.32     | 0.23     |
| 2-Deoxyglucose | 1.00    | 0.53    | 0.20     | 0.28     | 0.35     |

*Not determined.

| Table V |

Phosphotransferase activities in cells grown in the presence of nonmetabolizable glucose analogues

*S. typhimurium* SB3507 was grown in modified Medium 63 containing 0.4% DL-lactate plus 1 mM methyl α-glucoside or 2-deoxyglucose. Phosphotransferase activities were assayed as described in Table IV. Values are expressed as percentages of those obtained with cells grown in the absence of the glucose analogues.

| PTS activity | Analogue added to growth medium |
|--------------|--------------------------------|
|              | Methyl α-glucoside | 2-Deoxyglucose |
| *In vitro* activities |
| Enzyme I      | 138               | 118               |
| HPr           | 79                | 79                |
| IIMan         | 35                | 20                |
| Rate of transport |
| Methyl α-glucoside | 37               | 11               |
| 2-Deoxyglucose | 5                 | 5                 |

depend on growth conditions, the glucose receptors, IIMan and IIMan, can be repressed or derepressed. Based on the extreme cases (i.e. maximal repression and maximal derepression), the specific activities of the sugar-specific PTS protein complexes, IIIaMan and IIIaGlc, can vary about 15-fold.

Regulation of PTS Activities: Effect of Energy Sources—Another mechanism for regulating PTS activity in whole cells is by changing the energy levels in the cell. Others have shown (28) that inhibitors of the electron transport chain and of oxidative phosphorylation significantly increase the rate of uptake of methyl α-glucoside, presumably because the intracellular level of P-enolpyruvate from glycolysis is raised, although this explanation may be too simple and the phenomenon may be far more complex.

Uptake experiments were routinely conducted with cells washed free of exogenous catabolites. A series of experiments was therefore performed in which initial uptake rates and steady state levels of methyl α-glucoside were compared in the presence and absence of the carbon source used for growth of cells. The initial uptake rate was changed by varying the external sugar concentration available for transport.

Table VI shows the effect of varying the initial rate of methyl α-glucoside uptake by altering the concentration of the glycoside in the medium. Except for the lowest concentration used, 0.01 mM, which is ½ the Km for uptake, each of the steady state levels attained in these experiments ranged between 6 and 7 mM (internal concentration), despite the fact that initial uptake rates varied by 5-fold. The two most obvious explanations for these results are that the steady state level is determined by the total available pool of endogenous P-enolpyruvate reserves or that the PTS is highly regulated and the steady state concentration of internal methyl α-glucoside-P cannot exceed a certain value under a given set of conditions (strain, metabolic state of the cell, levels of PTS proteins, etc.).

Table VII shows the results of a study of methyl α-glucoside uptake in cells grown on various carbon sources and provides initial rates and steady state levels of uptake in the presence and absence of the carbon source used for growth and in the presence of the metabolic inhibitors cyanide, 2,4-dinitrophenol, and carbonyl cyanide m-chlorophenylhydrazine. The addition of inhibitors resulted in decreased rates of uptake in 11 of 12 different experiments; in only one was the rate decreased. These results therefore confirm (28) that inhibition of the electron transport chain or of oxidative phosphorylation has been published in a review (23), and is presented here in the Miniprint for the convenience of the reader.
results in more rapid uptake of methyl α-glucoside. These inhibitors also generally showed the expected results in the steady state levels of accumulation of methyl α-glucoside, in that, as the energy reserves of the cell were decreased by the action of the inhibitors, there was a concomitant reduction in the steady state levels of the glucoside. This was not observed for cells grown on glucose, presumably because the endogenous energy reserves are principally in the form of glycogen, and anaerobic glycolysis leading to P-enolpyruvate synthesis would not be affected by the inhibitors.

Table VII also shows uptake and steady state levels of methyl α-glucoside in cells grown on glycerol and with glycerol in the medium during transport experiments; similar experiments were performed with lactate-grown cells. The results on steady state levels of uptake suggest that these cells have low endogenous energy reserves, since, when they are supplemented with a utilizable carbon source, the steady state levels go up. When the carbon source (glycerol or lactate) was present in the medium during transport, however, there was a decrease in the initial rate of uptake of methyl α-glucoside.

The significance of these results is considered under “Discussion.”

In summary, it is clear from Tables IV and VII that the transport of solutes via the II*M* and II*G* systems in intact cells can be significantly affected in at least two ways. (a) The levels of the proteins may be changed by the growth conditions, and (b) the metabolic energy state of the cells may affect both initial rates and steady state levels of accumulation of solutes, and these two parameters may change in opposite directions.

**Assay of Mutant Strains Defective in II*G* and/or II*M***

Curtis and Epstein (8) showed that two classes of sugar-specific PTS mutants (glucose-specific, ptsG, and mannose-specific, ptsM) could be isolated from *E. coli*. The ptsM mutants could utilize glucose but could not utilize mannose or glucosamine, whereas the ptsG mutants showed reduced ability to use glucose, but mannose and glucosamine were less affected. Finally, double mutants containing both point mutations, which mapped in different areas of the *E. coli* genome, were unable to utilize any of the three sugars, whereas the utilization of mannitol (another PTS-sugar) was unaffected. Although the II*G*/II*G* system had been described earlier (29), Curtis and Epstein interpreted their results solely on the basis of the II-A/II-B system. In the present studies, the *E. coli* mutants were therefore re-investigated, together with a number of *S. typhimurium* mutants.

The results of these experiments are presented in Table VIII. The following three sets of characteristics were examined: the fermentation patterns on glucose and mannose, uptake properties with 2-deoxyglucose as a marker for II*M* and methyl α-glucoside as the marker for II*G*, and enzyme assays *in vitro*. In all cases, the three sets of results were consistent. The ptsG mutants can still ferment glucose and mannose and are defective in their ability to take up methyl α-glucoside. The ptsM mutants are unable to ferment mannose but can ferment glucose. The ptsM mutants contain reduced levels of II*M* and show a greatly reduced ability to take up 2-deoxyglucose. Mutants with defects in both genes, ptsG and ptsM, are unable to ferment glucose or mannose, contain reduced to negligible levels of II*G* and II*M*, and show greatly reduced abilities to take up both 2-deoxyglucose and methyl α-glucoside.

It is clear that the phenotype exhibited by pts mutants depends on the growth conditions before the transport (and perhaps even the fermentation) tests. Glucose can be taken up and utilized in an Enzyme I mutant via one of the galactose permeases (24) when the cells contain the ATP-requiring glucokinase. Similarly, 2-deoxyglucose (5) is a substrate of the specific galactose permease. Thus, it is very important to know the state of induction of the galactose permeases in pts mutants before interpreting any results. Furthermore, fructose, which can be utilized via the glucose PTS (23), can also induce the synthesis of a separate PTS specific for fructose (23).

Finally, as noted earlier in this paper, there appears to be an interrelationship, as yet undefined, between ptsG and ptsM, in that several mutants defective in one of the genes are also partially defective in the other. This relationship was not

### Table VIII

**Specific activities of PTS components in various mutant strains**

| Strain    | Relevant genotype |  
|-----------|-------------------|
|           | *In vitro* activities | *Transport rates* | *Fermentation* |  
|           | II*M* | II*G* | Methyl α-glucoside | 2-Deoxyglucose | Glucose | Mannose |  
| *E. coli* |  
| ZSC103    | ptsG  | ND*   | <4             | 8             | 6       | +       +      |  
| ZSC114    | ptsM  | ND    | <4             | 175           | 6       | +       +      |  
| ZSC112    | ptsG/ptsM|ND   | <4             | 175           | 6       | +       +      |  
| SB3507    | Wild type | 100 | 100 | 100 | 100 | + +       +      |  
| SB4004     | crr    | 0     | 13  | 36  | <2    | 17      | + +       +      |  
| SB3666     | ptsG  | 5     | <3  | 71  | <2    | 14      | + +       +      |  
| SB2263     | ptsG  | ND    | <5  | 61  | 4     | 40      | + +       +      |  
| SB4006     | ptsM  | ND    | 26  | 7   | 100   | 1       | + +       +      |  
| SB1687     | ptsM  | 50    | 113 | 18  | 141   | 3       | + +       +      |  
| SB3668     | ptsG/ptsM|15   | <3  | 18  | <2    | 3       | + +       +      |  

*Not determined.

*Similar results were obtained with strains SB4008 and SB4009.
consistent within each of the two sets of mutants, ptsG and ptsM, that were assayed and in some cases was more evident in the uptake experiments than in the in vitro assays. Of the three ptsG mutants, for example, each showed a slight reduction in the II'm activity (60-70% of wild type) but from 14-71% of the ability to take up 2-deoxyglucose. In practical terms, this unexplained and inconsistent behavior should be considered in characterizing ptsG and ptsM mutants.

**DISCUSSION**

In earlier papers, we had reported the presence of two PTS sugar-specific systems that phosphorylated glucose. One report described the separation of the proteins of the IIA/II-B system and their characterization (22). The other report described preliminary findings (29) showing the existence of the II'm/II-BG'C system. The isolation and characterization of the II'm system are presented in an accompanying paper (3).

The objective of the present study was to provide a clear definition of the characteristics of the two phosphotransferase systems, both in vivo and in vitro, and then to determine the biochemical lesions in mutants defective in one or the other (or both) of these transport systems. This required development of assays that could measure the activity of each system when both were present in the same membrane. With these assays, we were able to compare the kinetic properties and substrate specificities of the two systems and to measure the effects of growth conditions and energy levels on both systems in vivo and in vitro. The assays provided information on the biochemical defects in the mutants and clearly showed that regulation of PTS activities themselves and the regulatory effects of the PTS on other systems are complex processes.

The measurement of the II'm and II'BG'C systems depends on the relative quantities of HPr and II'B present in the assay system. Thus, with relatively high concentrations of HPr (20-40 mM) and no added II'B, the II'm system can be measured, whereas with endogenous HPr (or trace quantities of exogenous HPr) and 4 mM II'B, the II'B system operates and can be measured. We used these conditions to study the substrate specificities of the two systems. In accord with the previous results (8), we confirmed the presence of two phosphotransferase systems for glucose, ptsG with narrow specificity for glucose and methyl β-glucoside and ptsM with broader specificity for glucose, mannose, 2-deoxyglucose, glucosamine, and mannosamine. It is now clear that II'B activity is governed by ptsG, whereas the II'A/II-B (or II'm) system is governed by ptsM. The properties of the mutants are consistent with the lack of either II'BG'C or II'm. At this stage, we are unable to separate II'm from the II'A and II-B proteins with quantitative recovery of each, and thus, the II'm complex per se was measured in membranes in terms of its ability to phosphorylate 2-deoxyglucose. We would expect that there would be two subclasses of ptsM mutants, one defective in II'A and the other in II-BG'C. Furthermore, if, as suggested in an earlier study, there are II-A proteins specific for different sugars, there may be several subclasses of II-A mutants. In the present study, however, all Enzyme II'm mutants are considered in a single class.

With respect to the II'BG'C system, we can distinguish between the crr mutants which are deficient in III', as described in an accompanying paper (30), and the ptsG (II'BG'C) mutants described here.

Our studies have confirmed the sugar specificities of the two systems and also show that N-acetylmannosamine is a substrate, albeit a poor one, for the II'm system and that methyl β-glucoside can be phosphorylated by II'BG'C. Furthermore, the specificity of the two systems for their substrates does not appear to be absolute. For example, methyl α-glucoside can be phosphorylated by the II'm system, although the $K_m$ value is very high (25 mM) compared with 15 μM for glucose. In an analogous manner, high concentrations of mannose inhibited the uptake of methyl α-glucoside by the II'BG'C system. Both systems appear to be active with fructose; it is phosphorylated by II'm (Km, 20 mM) and although it was not phosphorylated by II'BG'C, high concentrations of fructose (Km, 30 mM) inhibited the uptake of methyl α-glucoside. Nevertheless, the large differences in kinetic values permitted a clear distinction of the ptsM and ptsG mutants in vivo and in vitro. It is interesting, however, that several mutants defective in one gene were partially defective in the products of the other. In addition, repression of both systems was brought about by growth of the organism on either methyl α-glucoside or 2-deoxyglucose. This suggests that a common element may be regulating the synthesis of the sugar-specific proteins, or perhaps a common polypeptide is involved in their function. Clearly, there is interaction between II'm and II'BG'C at some level, possibly at the level of transcription or translation or perhaps more directly at the level of transport. Although the properties of the mutants can be explained by the defects in II'm and II'BG'C, it is important to bear in mind that the phenotype exhibited by such mutants depends upon the growth conditions, and thus, the state of induction of other enzymes. For example, glucose can be taken up by non-PTS galactose permeases, and in the presence of glucokinase, 2-deoxyglucose can be phosphorylated and metabolized (5, 23, 24, 31). 2-Deoxyglucose is also transported by the specific galactose permease (5). Moreover, growth of the organism on specified sugars can either increase the amount of PTS proteins or repress their synthesis (see "Regulation of PTS Proteins: Effect of Growth Medium" under "Results"). The energy level in the cell also affects the activities of PTS proteins. As expected from previous studies (28), inhibition of the electron transport chain or oxidative phosphorylation increased the rate of uptake of methyl α-glucoside, presumably by increasing the concentration of P-enolpyruvate, although the steady state levels of methyl α-glucoside were reduced as the energy reserves were depleted. However, if the cells are grown on glycerol or lactate and the corresponding carbon source is present in the incubation medium when transport is measured, there is a decrease in the uptake rate of methyl α-glucoside. Since glycerol, which feeds into the glycolytic pathway, and lactate would be expected to increase the level of P-enolpyruvate, it would likewise be expected that the presence of these carbon sources would result in increased initial uptake rates of the glucone, rather than the reduced rates actually observed. Regulation here may, perhaps, be explained by the report of Mason et al. (32). The intracellular level of P-enolpyruvate in Streptococcus lactis is very high in starved cells and drops immediately upon the addition of glucose. Conceivably, a high initial rate of methyl α-glucoside transport requires a certain P-enolpyruvate concentration, and this may be low in cells exposed to glycerol or lactate. These results add further credence to the notion that the regulation of the phosphotransferase transport systems in the whole cell is an exceedingly complex process and may involve interactions between the PTS and the electron transport chain, as well as interaction among different sugar-specific proteins as described above.

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Sugar Transport by the Bacterial Phosphotransferase System. XXI

EXPERIMENTAL PROCEDURES

Materials - All sugars were of the D configuration and glucose was pyridoxal. The following radioactive compounds were obtained from New England Nuclear: D-glucose-1,2-14C (0.125 mCi/mmol); D-glucose-4,6-3H (tritiated, 15,000 cpm/mmol); D-glucose-6-3H (200,000 cpm/mmol). [U-14C] Glucose was purchased from DuPont-New Haven. Methyl-d-glucoside was synthesized from D-glucose as described previously (1). The methyl-d-glucoside was separated from methyl-C14-glucose by the method of Austin et al. (11). Absolute concentrations of the radioactive sugars were determined by exchange through loose column bonds (0.1 M NaCl, pH 2-3, 100-180 gpl).

Phosphoglucomutase, phosphoglucose isomerase, and several enzymes were purified by the method of Clark and Herlihy (15). Monosaccharides obtained from cells and cells free of sugars were rechromatographed from ethyl acetate, ethyl acetate:formic acid, and glucose or formic acid, and disodiumhydroxylate obtained from Nici. Beta-cellulose was the bezoar precipitated product of Nici. All other chemicals were reagent grade from standard commercial sources.

Bacterial strains - The bacterial strains were used in this study and pertinent reference information are given in Table I. E. coli (phosphatase strain alpha SB3659 and SB3666) were originally derived from SB3659 (yep21). SB3666 and SB3682 were derived from E. coli phosphatase LT2 (17).

Table I. Bacterial strains used in this study

| Strains     | Genotype | Parent | PTS defect | Genetic reference |
|-------------|----------|--------|------------|-------------------|
| SB3659      | alpha    | SB3541 | 1.0         |                   |
| SB3666      | alpha    | SB3682 | 1.0         |                   |
| SB3659 (yep21) |         |        |             |                   |
| SB3666 (yep21) |         |        |             |                   |
| SB3682 (yep21) |         |        |             |                   |
| SB3659 (yep1023) |         |        |             |                   |
| SB3666 (yep1023) |         |        |             |                   |
| SB3682 (yep1023) |         |        |             |                   |

The growth and collection of cells - Cells were grown in minimal salts media. Modified Medium A (18) and Medium A (11) were used interchangeably with no differences in transport or in cell uptake or phosphotransferase reaction; the media contained 0.18% of the indicated sugar or 0.125% MeCl.

Results - The experimental procedures were as follows: E. coli phosphatase SB3659 was grown to early stationary phase in minimal salts containing 0.125% MeCl. E. coli phosphatase LT2 was grown to milks salts plus 0.25 glucose in a fermentor at the National Institutes of Health, Bethesda, Maryland.

Table II. Initial intracellular concentrations of methyl-d-glucoside accumulation

| Initial intracellular concentration | 10^{-11} M | 10^{-10} M | 10^{-9} M | 10^{-8} M |
|-----------------------------------|------------|------------|-----------|-----------|
| 0.01                              | 0.1        | 0.1        | 0.1       | 0.1       |
| 0.02                              | 0.1        | 0.1        | 0.1       | 0.1       |
| 0.03                              | 0.1        | 0.1        | 0.1       | 0.1       |
| 0.04                              | 0.1        | 0.1        | 0.1       | 0.1       |
| 0.05                              | 0.1        | 0.1        | 0.1       | 0.1       |

Percentage of sugar initially added to cells which, after a steady state is reached, is located within the cells.
Table VII

Initial rates and steady state levels of methyl glucoside accumulation under different metabolic conditions.

| Condition | O.75 addition | 0.75 [H] addition | 0.75 [G] addition | 0.75 [L] addition |
|-----------|---------------|------------------|------------------|------------------|
| Initial steady state | rate | Initial steady state | rate | Initial steady state | rate | Initial steady state | rate | Initial steady state | rate |
| Methyl glucoside | 1.0 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| + 10 mg KCl | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |
| + 1 mg succinate | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 |
| + 10 mg carbon source | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 |

The results varied within the indicated range and appeared to depend on the presence or absence of NO in the medium.

[NO], not determined.

Sugar Transport by the Bacterial Phosphotransferase System. XXI

Fig. 1 - A, apparatus used to measure initial rates of transport: A, a weighed filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. B, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. C, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. D, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. E, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. F, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. G, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. H, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. I, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. J, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. K, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. L, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. M, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. N, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. O, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. P, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. Q, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. R, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. S, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. T, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. U, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. V, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. W, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. X, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. Y, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm. Z, a soluble filter paper note to an aluminum plate (12 cm x 12 cm x 0.125 cm). The holder is a stainless steel 200 x 200 x 200 cm.