Metabolism of D-Glucosamine and N-Acetyl-D-glucosamine in the Intact Rat*

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It has been demonstrated that D-glucosamine and N-acetyl-D-glucosamine are metabolized in animal tissues to D-fructose via phosphorylated intermediates (1-4). It has also been shown that D-glucose is converted to D-glucosamine and incorporated into glycoproteins and mucopolysaccharides (4-11), probably through formation of uridine diphosphate derivatives as intermediates (4). Little work has been done, however, to establish the quantitative significance of these pathways in the intact animal.

The present work represents a study of the metabolism of D-glucosamine, N-acetyl-D-glucosamine, and D-glucose in normal rats which were deprived of food, and is concerned with the comparative rates of oxidation, conversion to liver glycogen, incorporation as D-glucosamine and sialic acid into macromolecular liver components, and excretion in the urine.

EXPERIMENTAL PROCEDURE

Materials and Methods—The isotope-containing substances used were obtained from commercial sources or prepared synthetically as follows: D-glucose-1-C\text{14}, acetic-1-C\text{14} anhydride, and sodium acetate-1-C\text{14} were obtained from Volk Radio-Chemical Company. D-Glucosamine hydrochloride-1-C\text{14} was a gift from Pfizer Research Laboratory.

N-Acetyl-D-glucosamine-1-C\text{14} was prepared by acetylating D-glucosamine-1-C\text{14} with unlabeled acetic anhydride, by the method described by Roseman and Ludowig (12), but modified for use with millimole quantities. N-Acetyl-1-C\text{14}-D-glucosamine was prepared by acetylation of unlabeled D-glucosamine with acetic-1-C\text{14} anhydride according to the same method. In a typical preparation, 216 mg of D-glucosamine hydrochloride were dissolved in 5 ml of water and 0.5 ml of methanol. The solution was stirred at 0-5° with 6 ml of Dowex 1 (carbonate form) and 0.13 ml of acetic anhydride for 90 minutes. After filtering and washing, the solution was placed over 1 ml of Amberlite IR-120 (acid form), and the effluent was brought to a boil and concentrated under reduced pressure. The crude product was recrystallized as described (12), resulting in a yield of 73 mg (43\%) of N-acetyl-D-glucosamine melting at 211.5°.

Administration to Animals—The animals used in these studies were male Sprague-Dawley or Holtzman rats weighing 250 to 350 g. They were maintained on a stock diet and were deprived of food for 24 hours before use. In those experiments in which unlabeled carbohydrate was administered, the desired quantity of sugar or sugars was dissolved in 5 ml of water and administered by stomach tube. The isotope-containing substances was injected intraperitoneally, and the animal was immediately placed in a metabolism cage which enabled collection of respiratory CO\text{2} and urine. The amount of isotope administered varied somewhat in each experiment, but was approximately 2 μc of N-glucose-1-C\text{14}, 3 μc of sodium acetate-1-C\text{14}, 3 μc of N-acetyl-1-C\text{14}-D-glucosamine, and 4 μc of 1-C\text{14}-D-glucosamine or N-acetyl-1-C\text{14}-D-glucosamine. Either expired CO\text{2} was collected hourly for 4 hours, or a single 4-hour collection was made. At the end of that period, the animal was killed by a blow on the head and the liver was removed as rapidly as possible.

Isolation of Metabolic Products

Products resulting from the metabolism of the administered compounds were isolated, and isotope content was determined by methods which are described below.

1. Expired CO\text{2} and Urine—Respiratory CO\text{2} was trapped in carbon dioxide free 6 N NaOH. A suitable aliquot was diluted to prevent precipitation of Ba(OH)\text{2} and BaCO\text{3} was precipitated by addition of excess barium acetate. The precipitate was thoroughly washed with CO\text{2}-free water and counted at infinite thickness in an ultrathin window counter. Urine samples were removed from the cage after the 4-hour experimental period, the cage was washed with small volumes of water, and the combined urine and washings were diluted to 5 ml. Aliquots were acidified and brought to a boil to remove dissolved CO\text{2}. The solution was then oxidized to CO\text{2} by the Van Slyke-Folch wet combustion method (13) and counted in a Packard Tri-Carb liquid scintillation counter by the method of Jeffay and Alvarez (14).

2. Liver Glycogen—Glycogen was isolated from the livers of the experimental animals by several extractions with cold 5\% tri-chloroacetic acid in a Servall Omni-Mixer according to the method of Carroll, Longley, and Roe (15). The glycogen was oxidized to CO\text{2} by the Van Slyke-Folch method and counted as BaCO\text{3} at infinite thickness where possible. Correction to infinite thickness was made from a self-absorption curve in those cases in which insufficient glycogen was obtained.

3. Sialic Acid—The liver residue remaining after extraction of the glycogen was suspended in 0.3 N trichloroacetic acid, placed in a boiling water bath for 8 minutes, and then quickly cooled in an ice bath. Under these conditions, sialic acid is released from the precipitated protein, but D-glucosamine is not (11). The suspension was centrifuged, and trichloroacetic acid was removed by extraction with ether. Sialic acid was isolated...
from the solution with a gradient elution from Dowex 1 as described by Zilliken and O'Brien (18). The sialic acid content of the effluent was determined by the method of Warren (17). A known quantity of sialic acid was oxidized to CO₂ and counted in a liquid scintillation counter.

4. Hexosamines—After extraction of sialic acid, trichloroacetic acid was removed from the liver residue by washing with 95% ethanol. Hexosamines were liberated by hydrolysis for 10 hours in a boiling water bath with 3 N HCl, as described by Spiro (11). After filtration and removal of HCl by concentration to dryness in a vacuum, the residue was taken up in 15 ml of water and passed over a column containing 30 ml of Dowex 50-X4 resin prepared as described by Bone (15). The column was eluted with 2.0 N HCl or, in some cases, 0.5 N HCl, in an attempt to decrease the amino acid content of the effluent. The amino sugar content of the eluate was determined by the modified Elson-Morgan procedure described by Palmer, Smyth, and Meyer (19). The values reported presume no interference by the amino acids present. It was also found that the amino sugar was not quantitatively recovered from the column. Neither event, however, would interfere significantly with the interpretation of the data of this report.

Glucosazone was prepared from the D-glucosamine in the eluate by a modification of the method described by Shriner, Fuson, and Curtin (20). This preparation required considerable care in order to obtain a reasonably pure product. In our hands, the method described by Spiro (11) resulted in the formation of excessive amounts of tarry products which made purification by recrystallization difficult or impossible with the small amount of material available. All preparations were carried out by concentrating the eluate to a syrup in a centrifuge tube and adding sufficient D-glucosamine as carrier to bring the total content to 120 μmoles. To this were added 51.8 mg of recrystallized phenylhydrazine hydrochloride, 77.7 mg of sodium acetate·3H₂O, 0.2 ml of 50% NaHSO₃, and sufficient water to effect complete solution (0.6 to 1.0 ml). The tube was placed in a boiling water bath, a “cold finger” condenser was inserted, and, as an added inhibitor of oxidation, a stream of nitrogen was passed over the solution throughout the heating. Crystallization began after approximately 20 minutes. The tube was removed from the water bath when the first faint signs of darkening occurred, usually between 45 and 60 minutes of total heating time. The suspension was centrifuged and washed several times with cold water in the centrifuge tube. Recrystallization was best effected by dissolving the precipitate in a few drops of warm absolute ethanol, adding an equal volume of water, and placing the tube in an ice bath for 1 to 2 hours to promote crystallization. Recovery of the product was accomplished by centrifugation, on in a refrigerated centrifuge. The precipitate was washed with water and dried in a vacuum desiccator allowed to stand in a cold room. Yields of approximately 50% of product melting at 206–208° were obtained. A weighed amount of the osazone was oxidized to CO₂ and counted in a liquid scintillation counter. The percentage of administered radioactivity recovered a s protein-bound liver D-glucosamine was calculated from the specific activity of the isolated osazone and the amount of D-glucosamine in the eluate from the Dowex 50 column.

5. Liver Lipids—A crude lipid fraction was isolated from the livers of those animals used in experiments involving N-acetyl-1-C⁴-D-glucosamine and acetate-1-C⁴. Lipids were extracted from the liver by homogenization in a Servall Omnimixer once with 20 volumes of chloroform-methanol (2:1), and twice with 7 volumes of the solvent. The lipids were plated at infinite thickness and counted after removal of the solvent by evaporation in a vacuum oven at 60°.

In order to relate the counts recovered in any fraction to the counts administered in the compounds, the original tracer compounds were added to known amounts of like carrier compounds, oxidized to CO₂, and counted as BaCO₃ at infinite thickness and by the liquid scintillation method of Jeffay and Alvarez (14). Sufficient counts were taken in all instances to give a counting error of less than 5%. In counts made at infinite thickness in the thin window counter, the percentage of the administered radioactivity converted to each end product was calculated from the relationship

\[
\frac{\text{c.p.m. in administered compound (corrected to 26 mg/cm}^2) \times 100}{\text{c.p.m. in sample (corrected to 26 mg/cm}^2) - \text{c.p.m. in administered compound (corrected to 26 mg/cm}^2) \times 100}
\]

Correction to 26 mg per cm² was made from the relationship, c.p.m. X mg of sample/137, where the value of 137 represents the weight corresponding to 26 mg per cm² (total planchet area, 5.27 cm²).

RESULTS AND DISCUSSION

It is evident that approximately one-half of the tracer dose of glucose was converted to CO₂ in 4 hours (Group 1, Table 1), and that, as might be expected, addition of 1 or 2 g of D-glucose carrier reduced the percentage of tracer oxidized and increased the percentage found in liver glycogen (Groups 2 and 3, Table 1). Almost no liver glycogen was found in the absence of added carrier. Little more than 1% of the radioactivity from a tracer dose of D-glucose appeared in the urine.

Groups 4 and 5 of Table 1 show that much less of the D-glucosamine-1-C⁴ was oxidized to CO₂ or converted to liver glycogen, whether or not D-glucose was added as carrier, and that the presence of carrier did not alter the rate of oxidation. On the other hand, approximately one-half of the radioactivity from D-glucosamine appeared in the urine within 4 hours. This radioactivity was shown by chromatography and radioautography to be present as D-glucosamine. The excretion of large fractions of administered D-glucosamine has been noted previously in man (21, 22) and in rats (23) where large amounts of unlabeled D-glucosamine were administered.

The failure of D-glucosamine to be extensively oxidized, plus the fact that only a small fraction of the isotope from D-glucosamine was converted to liver glycogen even when D-glucose was added as carrier, indicates that the conversion of D-glucosamine to intermediates in the Embden-Meyerhof (glycolytic) pathway occurs only to a small extent.

After administration of N-acetyl-D-glucosamine-1-C⁴ (Groups 6 and 7, Table 1), however, oxidation to CO₂ was approximately 4 times as great as with D-glucosamine. Conversion to liver glycogen in the presence of D-glucose was 3-fold greater, with only 0.5 as much lost as urinary radioactivity, than was the case with D-glucosamine-1-C⁴. The urinary radioactivity was shown by chromatography and radioautography to be present as unchanged N-acetyl-D-glucosamine.

Experiments 8 to 11 of Table I were carried out to determine whether large amounts of D-glucosamine or N-acetyl-D-glucosamine had any influence on the metabolism of tracer D-glucose.
The amounts of isotope administered were approximately 2 mc of d-glucose-1-C\textsuperscript{14}, 3 mc of N-acetyl-d-glucosamine-1-C\textsuperscript{14}, and 4 mc of d-glucosamine-1-C\textsuperscript{14}. The animals were deprived of food for 24 hours before use. The experimental period was 4 hours.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Compound administered intraperitoneally</th>
<th>Compound administered orally</th>
<th>Glycogen obtained</th>
<th>Recovery of administered isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver glycogen</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>d-Glucose-1-C\textsuperscript{14}</td>
<td>None</td>
<td>s</td>
<td>0.63 ± 0.89</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>d-Glucose-1-C\textsuperscript{14}</td>
<td>d-Glucose (2)</td>
<td>9.4</td>
<td>7.1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>d-Glucose-1-C\textsuperscript{14}</td>
<td>d-Glucose (2)</td>
<td>20.9 ± 3.2</td>
<td>10.6 ± 1.6</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>d-Glucosamine-1-C\textsuperscript{14}</td>
<td>None</td>
<td>0</td>
<td>5.7 ± 1.5</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>d-Glucosamine-1-C\textsuperscript{14}</td>
<td>d-Glucose (2)</td>
<td>30.3 ± 4.3</td>
<td>5.3 ± 1.4</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>N-Acetyl-d-glucosamine-1-C\textsuperscript{14}</td>
<td>None</td>
<td>1.4 ± 1.4</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>N-Acetyl-d-glucosamine-1-C\textsuperscript{14}</td>
<td>d-Glucose (2)</td>
<td>23.1 ± 2.2</td>
<td>5.3 ± 1.4</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>d-Glucose-1-C\textsuperscript{14}</td>
<td>d-Glucose (1), d-glucosamine (1)</td>
<td>4.6 ± 1.9</td>
<td>3.3 ± 1.4</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>d-Glucose-1-C\textsuperscript{14}</td>
<td>d-Glucose (1), N-acetyl-d-glucosamine (1)</td>
<td>15.2 ± 8.9</td>
<td>9.9 ± 3.2</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>d-Glucose-1-C\textsuperscript{14}</td>
<td>d-Glucose (2)</td>
<td>0.46 ± 0.46</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>d-Glucose-1-C\textsuperscript{14}</td>
<td>N-Acetyl-d-glucosamine (2)</td>
<td>1.4 ± 1.9</td>
<td>0.6 ± 0.89</td>
</tr>
</tbody>
</table>

These experiments were suggested since Spiro (24) found an inhibition of d-glucose metabolism by liver slices in the presence of these amino sugars, and Rerabek (25) found inhibition of glucose utilization by kidney cells grown in culture. Analysis of the results of Experiments 10 and 11 compared with those of Experiment 1 indicates a statistically significant decrease in the amount of isotope recovered from d-glucose as CO\textsubscript{2} in the presence of d-glucosamine or N-acetyl-d-glucosamine. This may have been caused either by inhibition of d-glucose oxidation or by a dilution of the d-glucose pools due to conversion of the amino sugars to d-glucose metabolic intermediates. The two amino sugars, however, did not have significantly different effects.

The values shown in the last column of Table I were obtained by subtracting the sum of the radioactivity recovered from CO\textsubscript{2}, liver glycogen, and urine from 100. They indicate that approximately one-half of the administered isotope was not accounted for in the metabolic products mentioned above and must, therefore, have been either converted to macromolecular compounds or other metabolites or retained in the body as unchanged free sugars. The amounts of unchanged sugars were not determined.
TABLE III
Conversion of acetate-1-C14 and N-acetyl-1-C14-D-glucosamine to CO2 and liver lipids by normal rat

Approximately 3 μc of each isotope-containing substance were administered to each animal. The animals were deprived of food for 24 hours before use.

<table>
<thead>
<tr>
<th>Substance administered intraperitoneally</th>
<th>Recovery of administered isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expired CO2</td>
</tr>
<tr>
<td><strong>N-Acetyl-1-C14-D-glucosamine</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>33.4</td>
<td>0.14</td>
</tr>
<tr>
<td>45.4</td>
<td>0.33</td>
</tr>
<tr>
<td>36.2</td>
<td>0.43</td>
</tr>
<tr>
<td>(38.3 ± 5.1)*</td>
<td>(0.30 ± 0.12)</td>
</tr>
<tr>
<td>Sodium acetate-1-C14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>45.5</td>
<td>0.49</td>
</tr>
<tr>
<td>61.2</td>
<td>0.30</td>
</tr>
<tr>
<td>57.0</td>
<td>0.45</td>
</tr>
<tr>
<td>(54.5 ± 6.6)</td>
<td>(0.41 ± 0.08)</td>
</tr>
</tbody>
</table>

* Values in parentheses are means and their standard deviations.

The conversion of D-glucose to a host of compounds, is, of course, well known. Thus far, however, little is known of components to which D-glucosamine is converted. In view of the apparently limited conversion to glycolytic intermediates previously noted, it seemed likely that a significant proportion would be present as amino sugar components of the glycoproteins and acid mucopolysaccharides.

In order to determine the extent of this possibility, protein-bound hexosamine and sialic acid of liver were studied. The results of these studies are given in Table II. It is seen in this table that D-glucose gave rise to D-glucosamine and sialic acid of relatively low specific activity. D-Glucosamine, however, was an excellent precursor of macromolecular D-glucosamine and sialic acid of liver. N-Acetyl-D-glucosamine was a much better precursor than D-glucose, but much poorer than D-glucosamine.

The high specific activity found in liver macromolecular D-glucosamine and sialic acid when labeled D-glucosamine was administered is undoubtedly due, at least in part, to the presence of a much smaller pool of D-glucosamine than of D-glucose.

One of the questions raised by the differences in behavior between the metabolism of D-glucose and N-acetyl-D-glucosamine is the question of whether deacetylation of N-acetyl-D-glucosamine is a major reaction. That this is the case is indicated by the results shown in Table III, in which the conversions of N-acetyl-1-C14-D-glucosamine and acetate-1-C14 to expired CO2 and to liver lipids are compared. A large proportion of the radioactivity was converted to CO2, and appreciable amounts were found in liver lipids with both isotopes. A considerable proportion of the acetyl group of N-acetyl-D-glucosamine was oxidized to CO2, indicating that deacetylation of N-acetyl-D-glucosamine, or of a phosphorylated derivative, occurs extensively.

Determination of the extent to which tracer amounts of D-glucose, D-glucosamine, and N-acetyl-D-glucosamine are oxidized, excreted, or retained in the intact animal indicates (Fig. 1 and Table I) that glucose oxidation is most complete and that N-acetyl-D-glucosamine and D-glucosamine are less completely oxidized. As might be expected, excretion in the urine is in the inverse order. In all cases, approximately one-half of the tracer was retained in the animal at the end of 4 hours.

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**Fig. 1.** Rate of oxidation of D-glucose-1-C14 (●), D-glucosamine-1-C14 (○), and N-acetyl-D-glucosamine-1-C14 (△), as measured by recovery of administered isotope in expired CO2.

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**Fig. 2.** Interrelationships between the metabolism of the amino sugars and D-glucose. GNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine.
On the basis of previous reports (1–11), it appears likely that D-glucosamine is phosphorylated, converted to D-fructose 6-phosphate and metabolized, or converted to D-glucosamine or sialic acid nucleotides before incorporation into polysaccharides or glycoproteins. Present concepts of the interrelationships between the metabolism of the amino sugars and D-glucose are shown in Fig. 2. Many of these relationships have recently been reviewed (4). Assuming the essential correctness of this scheme, it appears from the results of Table I that the conversion of D-glucosamine to D-fructose 6-phosphate is not a favored pathway for D-glucosamine metabolism, and that N-acetyl-D-glucosamine is oxidized much more extensively than D-glucosamine. The converse is true in the reactions leading to incorporation into macromolecules, D-glucosamine being more extensively incorporated than N-acetyl-D-glucosamine. A more rapid phosphorylation of D-glucosamine than N-acetyl-D-glucosamine appears reasonable (26–28), which would account for the better incorporation of D-glucosamine into glycoproteins. The more rapid oxidation of N-acetyl-D-glucosamine can be explained by the observation of Comb and Roseman (3) and Leloir (2) that the deamination of D-glucosamine 6-phosphate is accelerated in the presence of N-acetyl-D-glucosamine. It seems likely that labeling will be limited largely to tissue components which become labeled, but in view of the lack of extensive mixing with intermediates of D-glucose oxidation, it seems likely that labeling will be limited largely to tissue components containing amino sugars. Presumably the amino sugar of components of tissues other than liver would also be labeled.

**SUMMARY**

D-Glucose-1-C14, D-glucosamine-1-C14, and N-acetyl-D-glucosamine-1-C14 were administered to rats which had been deprived of food, with and without D-glucose added as carrier, and the appearance of the isotope in expired CO2, urine, liver glycogen, protein-bound liver D-glucosamine, and sialic acid was determined. D-Glucosamine was not extensively oxidized to CO2 or converted to liver glycogen, and a considerable amount was excreted unchanged in the urine. However, there was extensive incorporation into trichloroacetic acid-precipitable D-glucosamine and sialic acid of the liver, suggesting that labeled D-glucosamine may be particularly useful in the study of glycoprotein and acid mucopolysaccharide metabolism.

D-Glucose served as a poor precursor of protein-bound D-glucosamine and sialic acid of liver, but was, of course, readily converted to liver glycogen and oxidized to CO2. N-Acetyl-D-glucosamine was intermediate between D-glucose and D-glucosamine in its oxidation, incorporation into macromolecules, and excretion.

Administration of N-acetyl-1-C14-D-glucosamine to rats which had been deprived of food resulted in recovery of the isotope in expired CO2 and liver lipids in amounts 70 to 75% of the amount recovered after administration of sodium acetate-1-C14, indicating a rapid deacetylation process.

**REFERENCES**
