THYMIDINE 5'-DIPHOSPHATE N-ACETYL-D-GLUCOSAMINE PYROPHOSPHORYLASE ACTIVITY OF HOG GASTRIC MUCOSA

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UDP-Linked N-acetylhexosamines occur in bacteria, plants, and animals and function as glycosyl donors in several enzymatic reactions in vitro. However, since ADP-N-acetyl-D-glucosamine occurs in plants (Dankert et al., 1964) and dTDP-D-glucosamine can be formed by bacterial extracts and further acetylated to dTDP-N-acetyl-D-glucosamine (dTDP-NAG) (Kornfeld and Glaser, 1962), it is probable that several nucleotides are involved in the metabolism of hexosamines. In the case of D-glucose, it has been shown that UDP-D-glucose gives rise to glycogen, ADP-D-glucose to starch (Leloir, 1964), and GDP-D-glucose to cellulose (Elbein et al., 1964). By analogy, then, N-acetylhexosamines linked to different nucleotides may possibly be destined for incorporation into different polysaccharides.

In connection with studies on the biosynthesis of soluble blood group substances (Kornfeld et al., 1964), extracts of hog gastric mucosa were tested for their ability to form possible sugar nucleotide precursors of mammalian heterosaccharides from N-acetyl-D-glucosamine-1-P and various nucleotide triphosphates. These extracts were able to form dTDP-NAG and epimerize this compound to dTDP-N-acetyl-D-galactosamine (dTDP-NAGal) in the presence of DPN. However, experiments in vivo also reported here, cast doubt on the physiological importance of these reactions.

EXPERIMENTAL

Fresh hog stomachs obtained from a local slaughter house were rinsed and the mucosal layer scraped off. The mucosa was then homogenized with 4 volumes of 0.025 M Tris-0.005 M $MgCl_2$ buffer, pH 8, in a glass

homogenizer with a motor-driven teflon pestle. After centrifugation at 15,000 x g for 15 minutes, the supernatant fluid was re-centrifuged at 85,000 x g for 1 hour. The second supernatant solution after passage over Sephadex G-25 was tested for its ability to form sugar nucleotides, as shown in Table I. By this method of assay, significant reactions occurred with UTP and dTTP. The reaction rates were linear for at least 1 hour. To confirm that the product was dTDP-NAG, a large-scale reaction was run and the product isolated by paper chromatography in solvent I (1 M ammonium acetate-95% ethanol (3:7.5)) and solvent II (1 M ammonium acetate, pH 3.8-95% ethanol (3:7.5)). The final yield of purified dTDP-NAG was 0.5 μmole. The isolated compound had chromatographic mobilities of authentic dTDP-NAG and exhibited the characteristic spectrum of thymidine.

TABLE I. Incorporation of NAG-1-P into nucleotides

Nucleotide	cpm Incorporated per reaction mixture	μmoles NAG-1-P Incorporated per g mucosa per hour
ATP	6.4	0.09
UTP	177.6	2.55
GTP	11.6	0.17
CTP	14.4	0.21
dTTP	48.0	0.69
dATP	5.6	0.08
dGTP	<2	<.03
dCTP	<2	<.03
none	<2	<.03

The reaction mixtures in a final volume of 0.25 ml contained 0.2 μmole C^{14} NAG-1-P, 800 cpm (donated by Dr. Paul O'Brien), 1.3 μmoles nucleotide triphosphate, 5 μmoles Tris, pH 8, 1 μmole $MgCl_2$, .9 μmoles mercaptoethanol, and 0.10 ml mucosal extract. After incubating at 37° for 1 hr., the reaction was stopped by boiling for 1 min. The unreacted NAG-1-P was hydrolyzed by a 2-hr. incubation with 0.25 mg of purified *E. coli* alkaline phosphatase (Worthington). Then 0.5 ml of a suspension of Dowex 1x8 formate (containing 65 mg of resin) was added to adsorb nucleotides; the Dowex was sedimented by centrifugation, washed twice with 5 ml of water, and the nucleotides eluted with 2 ml 4 N formic acid-0.4 N ammonium formate. 0.5 ml of the eluate was plated and counted in an end-window gas flow counter.

Chemical analysis revealed (per mole of deoxythymidine) 1.94 moles total phosphorus, 1.00 mole acid-labile phosphorus, and 1.02 moles of NAG. Hydrolysis (2 N HCl for 2 hours at 100°) of a C^{14}-labeled sample and subsequent column chromatography with authentic glucosamine and galactosamine (Gardell, 1953) revealed that all of the C^{14} label was associated with glucosamine. However, on re-incubation of dTDP-NAG-C^{14} with the mucosal extract in the presence of DPN, epimerization occurred and an equilibrium mixture of NAG and NAGal (78:22) was formed as shown by similar analysis. Unlike the bacterial system, the hog enzyme does not catalyze the formation of dTDP-glucosamine from dTTP and glucosamine-1-P under the conditions described in Table I.

Since extracts of hog gastric mucosa are capable of forming dTDP--N-acetylhexosamines, it was of interest to see if these compounds could be detected in hog stomach. A 50-pound hog was injected with 100 μc C^{14}--glucosamine in the left gastro-epiploic artery, and after 13 minutes the stomach was removed and its mucosa (85 g) extracted with 550 ml of boiling 70% ethanol. To the extract was added carrier, unlabeled dTDP-NAG and both dTDP-NAG and endogenous UDP-NAG were isolated from the extract by passage through a column of Sephadex G-25 and paper chromatography in solvents I, II and isobutyric acid-1 N NH_4OH (10:6). On assay of the purified sugar nucleotides for radioactivity, it could be calculated that the original extract contained 60,000 cpm as UDP-N-acetylhexosamine and less than 50 cpm as dTDP-N-acetylhexosamine. The latter figure is actually a maximum estimate as, at low levels of activity, significant contamination of the re-isolated dTDP-N-acetylhexosamine with extraneous radioactive material is likely. Ten grams of hog gastric mucosa contain 0.8 μmole UDP-N-acetylhexosamine and 0.12 μmole GDP-fucose. Assuming equal specific activities of N-acetylhexosamines in both UDP-NAG and the hypothetical dTDP-NAG, 10 g of mucosa could have, at most, 0.0007 μmole dTDP-N-acetylhexosamine, and probably less.

Even though steady-state concentrations of metabolic intermediates

can be misleading, the foregoing results suggest that, while extracts of hog gastric mucosa can readily make dTDP-N-acetylhexosamines from NAG-1-P and dTTP, both naturally occurring compounds, a deoxythymidine-linked pathway of hexosamine metabolism does not operate *in vivo*.

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