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EFFECT OF A SYNTHETIC HEXOSAMINE DERIVATIVE ON MUCOPOLYSACCHARIDE SYNTHESIS BY HUMAN CAPSULE AND SYNOVIUM*

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Abstract—A propionylated derivative of glucosamine (likely *N*-propionyl glucosamine) was prepared from propionic anhydride based on the method of preparation of *N*-acetyl glucosamine from acetic anhydride. This derivative inhibited, *in vitro*, the incorporation of labelled glucosamine or glucose into mucopolysaccharides (MPS) synthesized by human joint capsule and synovial tissue. The effect was similar to that observed when the anti-inflammatory agents, hydrocortisone or acetylsalicylic acid, were added to the system *in vitro*. These agents affect MPS synthesis through complex mechanisms. However, the inhibition by propionyl glucosamine appeared to be due to a specific effect on the incorporation of hexosamine precursors which could not be simply accounted for by pool dilution of the radioactivity.

PREVIOUS work from our laboratory has provided supportive evidence that cortisone, when given to animals in pharmacological doses, decreased the synthesis of mucopolysaccharides (MPS)‡ in several tissues.[1] At least part of that action appears to be mediated through a specific inhibition at the first step of the hexosamine synthetic pathway.[1,2] However, the effect of cortisone on protein synthesis[3] and glycolysis[4] tends to complicate a precise interpretation of this drug's action on MPS synthesis.[2] Because of this complexity of metabolic effects, it is not feasible to define clearly the exact mechanisms by which corticosteroids alter the inflammatory response of connective tissue. Furthermore, it becomes very difficult to determine what role the apparently accelerated synthesis of MPS[5] plays in the inflammatory response, since if glucocorticoids are used to study this problem there is a wide variety of other metabolic alterations that occur simultaneously.[3,4,6] The same argument applies to other anti-inflammatory drugs that have also been shown to suppress MPS or glycoprotein synthesis.[7–9] One approach to this problem could be through the use of a specific inhibitor of MPS synthesis, which would not be expected to interfere with glycolysis or protein metabolism. It was argued that, since the hexosamine pathway is unique for MPS (and glycoprotein) synthesis and since we had shown previously that at least one effect of cortisone was a suppression of this pathway,[1,2] then a synthetic hexosamine analogue might be expected to act as a suitable inhibitor. As the hexosamines of the MPS of animal tissues are largely in the *N*-acetylated form, it was argued that an *N*-propionylated form (i.e. a three- instead of a two-carbon *N*-derivative) might possibly

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‡ The terms mucopolysaccharides (MPS) and glycosaminoglycans (GAG) are used interchangeably.

serve as an appropriate inhibitor. Such a derivative was, in fact, prepared using a modification of the method of preparing *N*-acetyl glucosamine, as described under Methods.

The effect of this derivative was compared with the effects of two established anti-inflammatory agents (hydrocortisone and acetylsalicylic acid) on the synthesis of MPS by human connective tissues. The tissues were preincubated with the drugs in question, followed by the addition of radioactive precursors of the MPS. The effect on MPS synthesis was then determined by isolating the MPS and determining their specific activity.

METHODS AND MATERIALS

Preparation and characterization of the propionyl derivative of glucosamine

The method was based on the preparation of *N*-acetyl glucosamine from acetic anhydride:[10] 200 mg of glucosamine hydrochloride was dissolved in 60 ml water and 12 ml saturated sodium bicarbonate was added, followed by the addition of 12 ml of a 5 per cent solution of propionic anhydride (Eastman Kodak Company) in ethanol. The solution was stirred vigorously at room temperature for 5 min followed by heating at 100° for 3 min. It was then shaken at room temperature, with a mixed resin preparation (Dowex-50, 200–400 mesh, H^+ form, and Rexyn 201, 200–400 mesh, formate form; Fisher Scientific Co.), in a ratio of 2 : 1 until all gas evolution had ceased. The solution was then allowed to pass through a 20×1 cm column prepared by carefully packing a resin mixture of the above-mentioned composition. The column was washed with approximately 4 vol. of water and the combined eluates were flash-evaporated at 40° to near-dryness.

For analytical studies, the material was taken up in a minimal amount of hot ethanol from which a white solid crystallized at room temperature. Crystallization was completed by allowing the mixture to stand overnight at 5° (yield, 62 per cent). Elemental analysis showed: C, 46·25; H, 7·15; N, 6·50% (calculated: C, 45·99; H, 7·23; N, 5·99%). A portion of this material was also subjected to mass spectral analysis using a double-beam M5-30 mass spectrometer and a gradually increasing temperature range from 200° to 285°. Although a molecular ion could not be readily identified, peaks at 72 and 100 a.m.u. were consistent with a propionyl-substituted secondary amine that would result from beta cleavage of a longer chain.

The derivative was readily soluble in water and was routinely assayed with the acetic anhydride modification of the Elson–Morgan reaction as it is used in the differential assay of hexosamines and *N*-acetyl hexosamines.[11] With this method the propionylated material gave an absorption spectrum identical to that of *N*-acetyl glucosamine, as it also did with the acetyl acetone procedure.[11] With the acetic anhydride method, the color yield for the propionylated glucosamine was the same whether or not the acetylation step with acetic anhydride was carried out, as was the case with *N*-acetyl glucosamine. This method gave a 97–103 per cent conversion of glucosamine into the propionylated derivative with respect to the parent glucosamine hydrochloride. The derivative could be clearly separated from *N*-acetyl glucosamine on paper chromatography in a butanol–acetic acid–water system (see Fig. 1), or in an isobutyric acid–water system,[12] as a single spot. In order to test the purity of the derivative further, ^{3}[H]D-glucosamine (sp. act., 0·16 Ci/mg) was used for preparing the propionylated derivative as described above. The product, which had the same specific activity as the

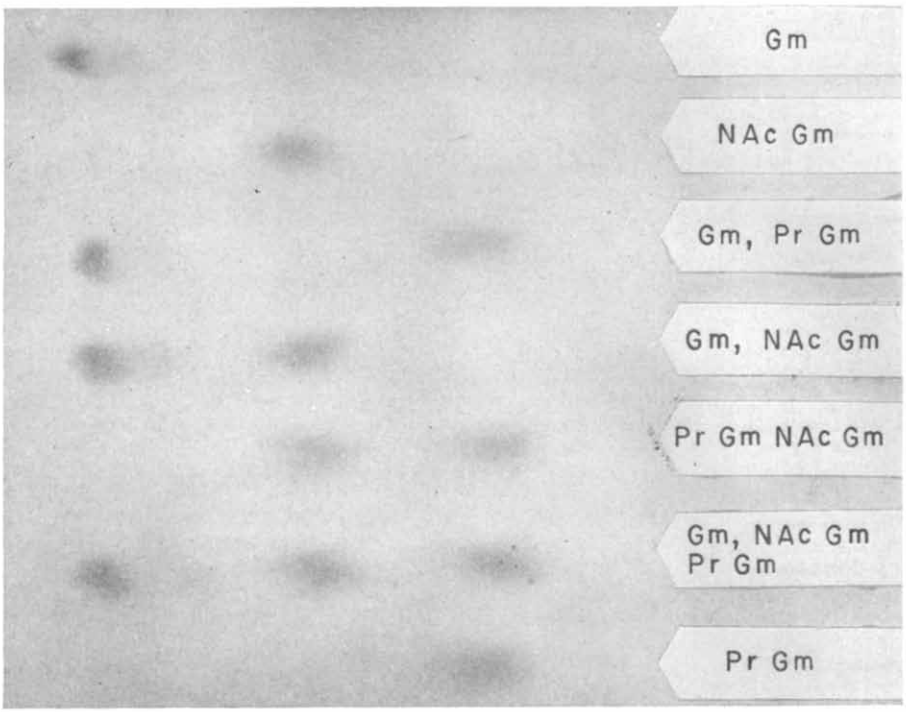

FIG. 1. Chromatographic separation of propionyl glucosamine from *N*-acetyl glucosamine and glucosamine. Paper chromatography was used as described under Methods. *N*-acetyl glucosamine and the propionyl glucosamine derivative gave a purple spot with the Elson–Morgan spray. Glucosamine gave a bright pink spot. The origin is at the left edge of the chromatogram and the solvent front at the right margin. Abbreviations are as follows: Gm, glucosamine; NAc Gm, *N*-acetyl glucosamine; Pr Gm, propionyl derivative of glucosamine.

^{3}[H]glucosamine (based on the acetic anhydride modification of the Elson–Morgan reaction[11]), was subjected to paper chromatography, the chromatogram was cut widthwise into 1-inch strips, and the radioactivity in each section estimated. Only one radioactive peak could be detected for the propionylated derivative (see Fig. 2).

Tissue incubation experiments

Source of connective tissues. Tissue was obtained from patients' joints at arthrotomy and transported at once to the laboratory at 0°. Most of the samples were derived from hip joint capsule from patients undergoing total hip replacement for osteoarthritis. Some "rheumatoid" synovium was obtained during synovectomy of the knee from patients with rheumatoid arthritis. Small amounts of "normal" synovium were also obtained during the removal of damaged semilunar cartilages. None of the patients was on corticosteroids for therapy, although some of the patients had been taking salicylates before surgery. Routine histology was done for all tissues.

Incubation procedure and isolation of the MPS. The tissues were cut into small pieces, which were pooled, and about 600-mg portions were weighed out of this pool. The tissue sample was added to 5 ml of modified Krebs–Ringer phosphate–glucose (0·154 N NaCl, 100 ml; 0·154 M KCl, 4 ml; 0·11 M $MgSO_4$, 1 ml; 0·154 M $NaHCO_3$, 3 ml; 0·1 M phosphate buffer, pH 7·4, 10 ml; 0·11 M $CaCl_2$, 2 ml; glucose, 1 mg/ml), which also contained 1000 i.u. of penicillin G. Preincubation with the drugs in question was carried out at 37° for 1 hr. The labelled MPS precursors were then added and incubation was carried on at 37° for the time periods indicated; the reaction was stopped by boiling for 3 min.

The MPS were isolated following a modification of the procedure of Scott[13] and of Antonopoulos and Gardell.[14] Briefly, a papain digestion of the whole incubation mixture was carried out, followed by centrifugation and extensive dialysis of the supernatant against water. The dialysant was again centrifuged and the supernatant was adjusted to 0·03 M with NaCl. Precipitation of the MPS was then carried out by adding dropwise a 5% solution of cetylpyridinium chloride (CPC) in 0·03 M NaCl. The precipitate was allowed to aggregate for several hours at 37° and collected by centrifugation. The CPC–MPS complex was dissolved in 2 ml of 2·0 M $CaCl_2$ at 37° with continuous shaking. The solution was then centrifuged and the MPS precipitated from the supernatant with 5 vol. of absolute ethanol in the cold. The precipitate was collected by centrifugation, again dissolved in 2·0 M $CaCl_2$, and the ethanol precipitation repeated. The precipitate was then dissolved in water and aliquots of this clear solution were used for estimation of hexuronic acid.[15] The radioactivity was estimated by applying 0·1-ml aliquots to 2·5 × 4 cm strips of filter paper, which were dried, placed in vials, and counted[16] in a Phillips liquid scintillation counter. The effects of the orientation of the paper strips with respect to the photomultiplier tubes were studied and, in agreement with previous authors,[16] were not found to alter significantly the counting efficiency, which was 33·4 per cent for tritium and 65·7 per cent for ^{14}C. All samples were counted for a sufficient time to give less than 5 per cent standard error in net counting rate.[17]

Recoveries of hyaluronic acid and chondroitin sulfate were approximately 100 per cent if the MPS standards were directly precipitated with CPC from 0·03 M NaCl, and 80 per cent for hyaluronic acid and 98 per cent for chondroitin sulfate if the MPS

were added at the beginning of an incubation, in the presence of tissue as "internal standards."

6-^{3}[H]D-glucosamine and 1-^{14}[C]D-glucose were obtained from Amersham–Searle and New England Nuclear Corp. respectively. Cetylpyridinium chloride was obtained from Sigma Chemicals Co.

RESULTS

Figure 1 illustrates the chromatographic separation[12] of *N*-acetyl glucosamine and glucosamine from the propionylated glucosamine derivative. It is noteworthy that both the propionylated and acetylated glucosamine derivatives give a purple spot.

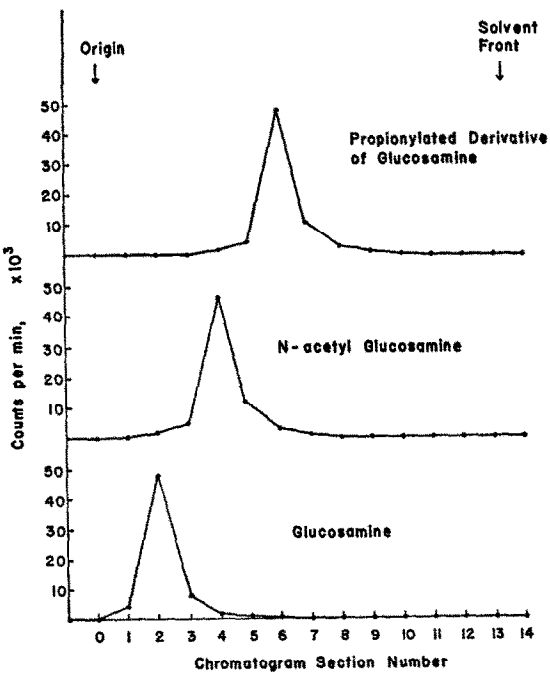

FIG. 2. Chromatographic separation of ^{3}[H]propionyl glucosamine. Paper chromatography was carried out as described under Methods, following the application of approximately 70,000 cpm of ^{3}[H]glucosamine, ^{3}[H]*N*-acetyl glucosamine and propionylated ^{3}[H]glucosamine (specific activities adjusted at 0·16 Ci/mg).

while free glucosamine gives a cherry red spot. A clean separation was achieved from *N*-acetyl glucosamine and glucosamine. There was no evidence of incomplete conversion of the propionylated derivative or of significant amounts of other products. A similar separation was achieved using an isobutyric acid–water (1:1) system.[12] When the tritiated propionylated derivative was subjected to paper chromatography (Fig. 2), only a single peak was again seen, and counts before and after the peak were at background levels.

Figure 3 shows the rate of incorporation of ^{14}[C]glucose and ^{3}[H]glucosamine into the isolated MPS synthesized by tissue from human osteoarthritic joint capsule. It can be seen that the specific activities continue to rise until approximately 48 hr and

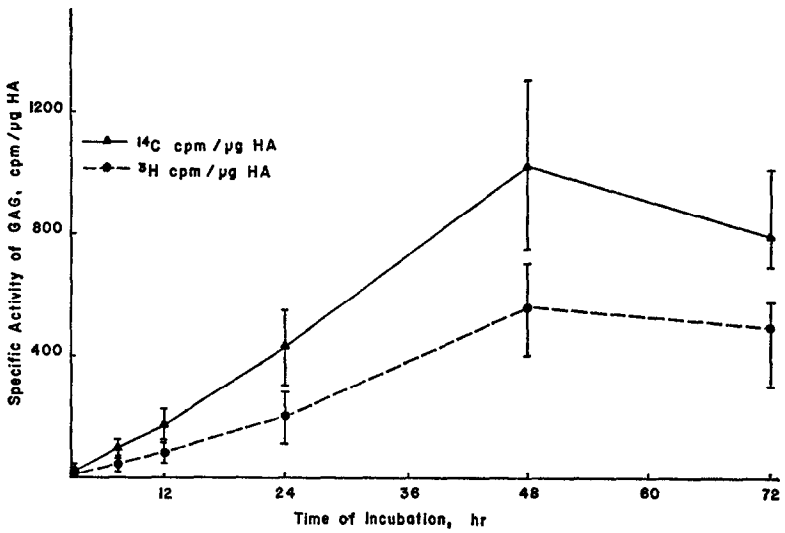

FIG. 3. Incorporation of radioactive precursors into MPS synthesized by human joint capsule. One μCi ^{3}[H]glucosamine (sp. act., 10 mCi/m-mole) and 5 μCi ^{14}[C]glucose (sp. act., 3·5 mCi/m-mole) were added to the incubation mixture and the reaction was stopped at the times shown. The MPS were isolated as indicated under Methods.

then appear to fall off. A 24-hr incubation period was therefore used routinely for most of the subsequent experiments.

The effects of two established "anti-inflammatory" drugs (hydrocortisone and acetylsalicylic acid) were compared with those of the propionylated glycosamine derivative on the incorporation of ^{3}[H]glucosamine and ^{14}[C]glucose into MPS synthesized by osteoarthritic human joint capsule (Fig. 4). It can be seen that both hydrocortisone and acetylsalicylic acid, added in high concentration, cause a pronounced decrease in the incorporation of MPS precursors after 24 hr of incubation, as did the propionylated glucosamine derivative.

Essentially similar results, with a smaller number of experiments, were obtained after 48 hr of incubation (Table 1). The inhibition in incorporation by propionyl glucosamine did not appear to be due to the propionic acid moiety *per se*, since sodium propionate, at the same concentration as propionyl glucosamine, had no effect on this system.

This effect with the propionylated glucosamine derivative did not seem to be tissue specific, as the decrease in incorporation was also observed with a sample of "normal" synovium and with synovium from patients with rheumatoid arthritis (Table 2).

The effect of various concentrations of propionyl glucosamine on the incorporation of labelled precursors was studied and Fig. 5 illustrates the decrease in incorporation of ^{14}[C]glucose and ^{3}[H]glucosamine into the isolated MPS from human "osteoarthritic" joint capsule with increasing concentrations of propionyl glucosamine. It is apparent that there was less ^{3}[H]glucosamine and ^{14}[C]glucose incorporated into isolated MPS with increasing concentrations of propionylated glucosamine.

However, one of the questions that arose was whether the decrease in incorporation noted after the addition of propionyl glucosamine may have been simply due to a "pool

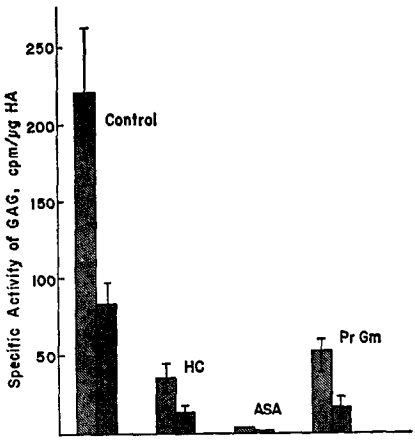

FIG. 4. Effect of some drugs on the incorporation of radioactive precursors into MPS synthesized by human joint capsule. The drugs were added to the incubation mixture at the following concentrations (μmoles/ml): hydrocortisone semisuccinate, disodium salt (HC), $4{\cdot}1 \times 10^{-3}$ M; acetyl salicylic acid (ASA), $5{\cdot}5 \times 10^{-3}$ M; propionylated glucosamine (Pr Gm calculated as *N*-propionyl glucosamine), $4{\cdot}2 \times 10^{-3}$ M. The drugs were preincubated with the tissue and buffer for 1 h. One μCi ^{3}[H]glucosamine (10 mCi/m-mole) and 5 μCi ^{14}[C]glucose (3·5 mCi/m-mole) were then added and the reaction was stopped 24 h later. The hatched bars represent ^{14}C label and the stippled bars represent ^{3}H label incorporated into the MPS isolated as indicated under Methods. Standard errors are indicated by the solid lines on the bars. Each bar represents the mean of at least four experiments, each carried out with tissue from a different individual, except for the ASA values which represent the mean of two experiments.

dilution" phenomenon rather than to an actual inhibition of incorporation of substrate. This could occur if, for example, propionyl glucosamine were depropionylated during the incubation. In order to answer this question, the effect of propionyl glucosamine on the incorporation of radioactive precursors was compared to that of *N*-acetyl glucosamine and glucosamine (Fig. 6).

It is apparent that, although the addition of "cold" glucosamine and *N*-acetyl glucosamine caused a decrease in the incorporation of ^{3}[H]glucosamine and ^{14}[C]-

TABLE 1. EFFECT OF SOME DRUGS ON THE INCORPORATION OF RADIOACTIVE PRECURSORS INTO MPS SYNTHESIZED BY HUMAN JOINT CAPSULE*

Addition	Time of incubation (hr)	Sp. act. of isolated GAG (cpm/μg hexuronic acid)	
		^{14}C	^{3}H
None	48	600	200
HC	48	70	20
ASA	48	3	0
Pr Gm	48	40	10

* Concentrations of drugs, amounts and specific activities of radioactive precursors, and conditions of incubation were identical to those of Fig. 4; however, the incubation time was 48 hr.

TABLE 2. EFFECT OF PROPIONYL GLYCOSAMINE (PR GM) ON THE INCORPORATION OF RADIOACTIVE PRECURSORS INTO MPS SYNTHESIZED BY HUMAN SYNOVIAL TISSUE*

Type of tissue	Drug added	Time of incubation (hr)	Sp. act. of isolated GAG (cpm/μg hexuronic acid)	
			^{14}C	^{3}H
Normal	None	24	250	70
Synovium	Pr Gm	24	30	10
Rheumatoid	None	24	350	140
Synovium	Pr Gm	24	25	6

* Conditions of the incubation, the concentration of Pr Gm, and amounts and specific activities of radioactive precursors were as in Fig. 4.

glucose into the isolated MPS, the decrease caused by an identical concentration of the propionylated derivative was considerably greater.

The incorporation of label in the hexosamine moiety of the isolated MPS was also studied by acid hydrolysis of the MPS,[1] followed by isolation of the hexosamines on a Dowex-50 column.[18] The specific activity of the hexosamine (cpm/μg hexosamine)

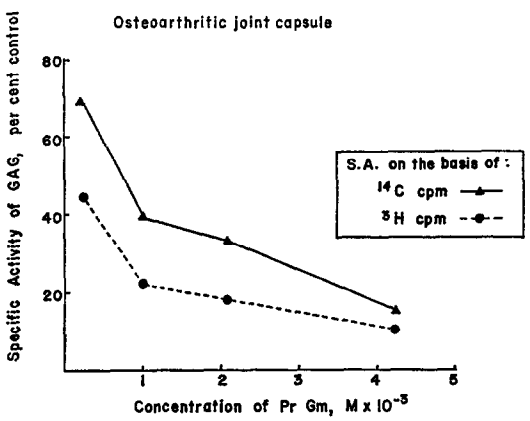

FIG. 5. Effect of concentration of propionyl glucosamine (Pr Gm) on the incorporation of radioactive precursors into MPS synthesized by human joint capsule. Conditions of the experiment were as indicated in Fig. 4.

from control experiments was 43·8 for ^{14}C counts and 5·9 for ^{3}H counts, while the specific activity of the hexosamine of the MPS synthesized in the presence of propionylated glucosamine was 15·9 for ^{14}C and 2·5 for ^{3}H. Clearly, the incorporation of both labels into the hexosamine of the MPS synthesized in the presence of propionyl glucosamine was considerably reduced compared to that of controls.

In order to investigate further the question of "pool dilution", ^{3}[H]glucosamine, ^{3}[H]*N*-acetyl glucosamine and ^{3}[H]propionyl glucosamine of equal specific activities were prepared as described under Methods, and the incorporation of these materials into the isolated MPS was compared (Table 3).

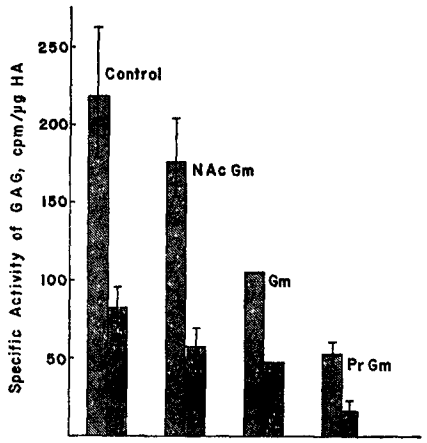

FIG. 6. Effect of some hexosamine derivatives on the incorporation of radioactive precursors into MPS synthesized by human joint capsule. Conditions of the experiments were as indicated in Fig. 4. All the amino sugars were at a concentration of 4×10^{-3} M.

Although both ^{3}[H]glucosamine and ^{3}[H]*N*-acetyl glucosamine were incorporated into the isolated MPS, there was only very slight, if any, incorporation of the ^{3}[H]propionyl glucosamine derivative. Furthermore, paper chromatography[12] of the supernatant of the tubes that contained propionyl glucosamine failed to reveal any breakdown products of this material (by either radioactivity or a colour spot on the chromatogram) at the end of the incubation period, while several radioactive products could be identified in the tubes that contained ^{3}[H]glucosamine or ^{3}[H]*N*-acetyl glucosamine.

TABLE 3. COMPARISON OF THE INCORPORATION OF ^{3}[H]PROPIONYL GLUCOSAMINE WITH ^{3}[H]N-ACETYL GLUCOSAMINE AND ^{3}[H] GLUCOSAMINE INTO GAG*

Radioactive material added	Sp. act. of isolated GAG (cpm/μg hexuronic acid)
Glucosamine	8
N-acetyl glucosamine	6
Propionyl glucosamine	1

* All the radioactive materials had been adjusted to a specific activity of 0·16 μCi/mg and were at a final concentration of 1 mg/ml of incubation mixture. Conditions of incubation and isolation of the GAG were as indicated under Methods.

DISCUSSION

The present work indicates that a propionylated derivative of glucosamine inhibits the incorporation of radioactive precursors into mucopolysaccharides synthesized by human connective tissue. From the analytical data presented, it would appear that

this derivative has a structure compatible with *N*-propionyl glucosamine. Both the colourometric properties and the method of preparation[10] are similar to that for *N*-acetyl glucosamine, but the propionylated derivative can be clearly distinguished by paper chromatography (Figs. 1 and 2). Although it is conceivable that propionyl substitutions may have occurred at other sites besides the 2-amino-2-deoxy, so that small amounts of other products would be formed, this seems unlikely from the paper chromatography experiments with the tritiated derivative (Fig. 2). In these experiments one would expect to detect even small amounts of additionally propionylated products with different R_f values, which might not be visualized with the nonradioactive preparation (Fig. 1). However, all of the radioactivity corresponded with the Elson–Morgan positive spots. Furthermore, the complete recovery (97–103 per cent), in terms of *N*-acetyl glucosamine equivalent, and the expected lability of *O*-acyl substitutions[19] when heated in an alkaline medium (see Methods) would also argue against more than one propionyl substituted product in significant amounts.

Both ^{3}[H]glucosamine and ^{14}[C]glucose have been used in the present work for evaluating incorporation of precursors into MPS. Glucosamine enters the hexosamine synthetic pathway below the first step (i.e. the amidotransferase reaction) either by being phosphorylated or by being first acetylated and then phosphorylated,[20] as shown in Fig. 7. ^{14}[C]glucose label is expected to enter via the amidotransferase

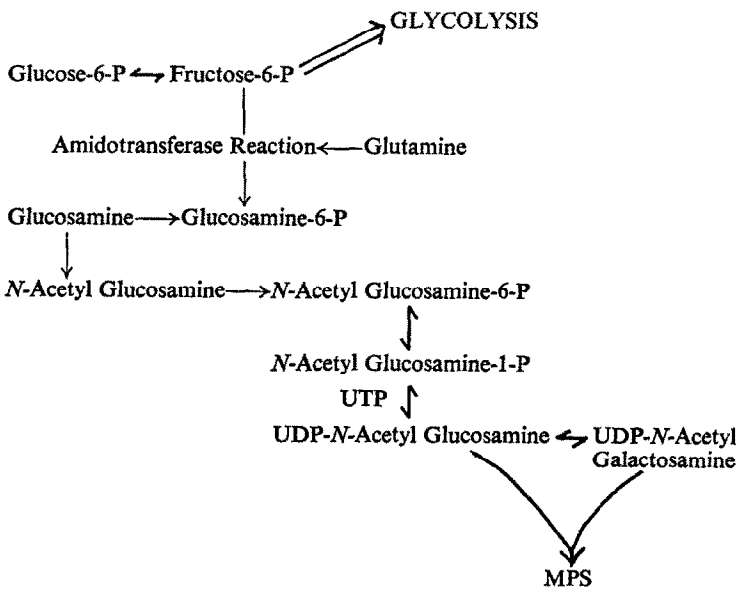

FIG. 7. The hexosamine synthetic pathway.

step, although the ^{14}C label in the isolated MPS would be expected to reflect also the counts incorporated into glucuronic acid.

In the incubation system used, the primary source of the carbon chain in both the hexosamine and glucosamine acid moieties in the synthesized MPS would be derived from glucose,[21] which had been added in substrate amounts, resulting in a significant pool dilution of the ^{14}[C]glucose. However, ^{3}[H]glucosamine, after being acetylated or

phosphorylated (Fig. 7), would also be expected to undergo pool dilution at the glucosamine-6-P and the *N*-acetyl glucosamine-6-P steps, since the carbon skeleton of most of these molecules would also be derived from glucose.

In this work the results have been calculated as specific activities of the total isolated MPS in order to minimize analytical variability. Nevertheless, the increase in specific activity with time (Fig. 3) likely reflects the rate of synthesis of MPS, at least during the initial part of the curve, and the 24-hr incubation was chosen as a convenient time interval. With very prolonged incubation times, a tendency of the specific activities to drop off was noted (Fig. 3). The reason for this is unclear but may be, in part, related to glycogenolysis in the synovium[22] and a subsequent decrease in the specific activity of the labelled glucose substrate.

The effect of propionylated glucosamine in inhibiting radioactive precursor incorporation into MPS synthesized by human connective tissue *in vitro* was observed at relatively high concentrations of inhibitor (Figs. 4, 5, 6, and Tables 1 and 2). For example, a 50 per cent inhibition of ^{3}[H]glucosamine label into MPS was noted at a concentration of propionylated glucosamine approximately one-tenth the concentration of the glucose substrate in the medium (Fig. 5). In these experiments it is conceivable that the propionylated glucosamine could be depropionylated to glucosamine, thus reducing the specific activity of the added ^{3}[H]glucosamine. The specific activity of the ^{14}[C]glucose label entering into hexosamine pathway intermediaries would then also be expected to decrease because of intermediary pools shared by the two labels, below the amidotransferase step (Fig. 7), resulting in lowered specific activities of the isolated MPS. However, this possibility of a pool dilution effect is rendered improbable by the results shown in Fig. 6, where the decrease in incorporation of the two labels into MPS was greater in the incubation tubes that contained propionylated glucosamine than in tubes that contained glucosamine or *N*-acetyl glucosamine, and by the observation that ^{3}[H]propionylated glucosamine was not converted into either glucosamine or *N*-acetyl glucosamine during the incubation, as shown by the paper chromatography experiments. Furthermore, ^{3}[H]propionylated glucosamine is apparently not incorporated to any significant extent into MPS (Table 3).

The exact enzymatic site of action of the propionylated glucosamine derivative in inhibiting incorporation of precursors (and presumably synthesis of MPS) needs further work in order to be elucidated. Nevertheless, it is noteworthy that both ^{14}C and ^{3}H label incorporation into the isolated hexosamine from the MPS was reduced by about the same proportion. This would suggest that the site of inhibition is below the amidotransferase step and probably below the acetylation of glucosamine-6-P (Fig. 7), since it appears that the major proportion of free glucosamine enters the hexosamine synthetic pathway by first being acetylated rather than by initial phosphorylation and then acetylation.[20] Both cortisone[1,2] and acetylsalicylic acid[7] appear to have complex mechanisms of action, the effect of salicylate inhibition being, at least in part, due to an inhibition of UDP-*N*-acetyl glucosamine–UDP-*N*-acetyl galactosamine epimerase,[23] although a direct inhibition of the amidotransferase reaction has also been demonstrated.[24] The use of specific inhibitors should help clarify the importance of the relative effects of anti-inflammatory agents on the carbohydrate and protein components relating to the synthesis of protein–polysaccharide, and may also be used as a tool to study the role of these substances in the inflammatory response.

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REFERENCES

1. T. Anastassiades and D. Dziewiatkowski, *J. lab. Clin. Med.* **75**, 826 (1970).
2. T. Anastassiades, *Biochim. biophys. Acta* **244**, 167 (1971).
3. J. Haroune and P. Feigelson, *Biochim, biophys. Acta* **199**, 214 (1970).
4. J. Ashmore and G. Weber, in *Carbohydrate Metabolism and its Disorders* (Eds. F. Dickens, P. J. Randle and W. J. Whelan), p. 358. Academic Press, London (1968).
5. C. W. Castor, R. K. Prince and M. J. Hazelton, *Arthritis Rheum.* **9**, 783 (1966).
6. C. Ottolenghi and O. Barnabel, *Endocrinology* **86**, 949 (1970).
7. M. W. Whitehouse and H. Bostrom, *Biochem. Pharmac.* **11**, 1175 (1962).
8. D. A. Kalbhen, K. Karzel and R. Domenjoz, *Med. Pharmac. exp.* **16**, 185 (1967).
9. J. Musil, J. Weissova, M. Adam and J. Prokopec, *Pharmacology* **1**, 295 (1968).
10. S. Roseman and I. Dafner, *Analyt. Chem.* **28**, 1743 (1956).
11. E. A. Davidson, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. VIII, p. 58. Academic Press, New York (1966).
12. S. M. Partridge, *Biochem. J.* **42**, 238 (1948).
13. J. E. Scott, in *Methods of Carbohydrate Chemistry* (Eds. R. L. Whistler), Vol. V, p. 38. Academic Press, New York (1965).
14. C. A. Antonopoulos and S. Gardell, *Acta chem. scand.* **17**, 1474 (1963).
15. Z. Dische, *J. biol. Chem.* **167**, 189 (1947).
16. C. H. Wang and D. E. Jones, *Biochem. biophys. Res. Commun.* **1**, 203 (1959).
17. C. H. Wang and D. L. Willis, *Radiotracer Methodology in Biological Science*, pp. 186–209. Prentice-Hall, New Jersey (1965).
18. N. F. Boas, *J. biol. Chem.* **204**, 553 (1953).
19. D. Horton, in *The Aminosugars* (Ed. R. W. Jeanloz), Vol 1A, p. 13. Academic Press, New York (1969).
20. J. F. McGarrahan and F. Maley, *J. biol. Chem.* **237**, 2458 (1962).
21. E. A. Davidson, in *The Aminosugars*, (Eds. E. A. Balazs and R. W. Jeanloz), Vol. II B, p. 2. Academic Press, New York (1966).
22. D. P. Page Thomas and J. T. Dingle, *Biochem. J.* **68**, 231 (1958).
23. H. Nakagawan and J. P. Bentley, *J. Pharm. Pharmac.* **23**, 399 (1971).
24. B. Jacobson and H. Bostrom, *Biochim. biophys. Acta* **83**, 152 (1964).