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CANINE THYROID FUCOKINASE

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Summary

A radiometric assay was developed for fucokinase (ATP:6-deoxy-L-galactose 1-phosphotransferase, EC 2.7.1.52) based on the conversion of L-[^{14}C]fucose to L-[^{14}C]fucose 1-phosphate which is trapped and counted on ion exchange paper. This assay was used to detect the presence of a fucokinase in canine thyroid tissue which was subsequently purified 2754-fold over the crude tissue extracts. The product of the fucokinase was identified as the β-anomer. The pH versus activity curve for the enzyme appears biphasic with optima at pH 6.5 and pH 8.25. The enzyme was shown to be highly specific for L-fucose with a K_m of $2.6 \cdot 10^{-5}$ M at pH 8.25. It was shown to be absolutely specific for ATP as a phosphate donor with a K_m of $6.3 \cdot 10^{-4}$ M at pH 8.25. The enzyme requires a divalent cation. Mg^{2+} is slightly more effective than Mn^{2+} in meeting this need. The molecular weight of the enzyme has been determined to be 494 000 ± 12 400.

Introduction

A classical study by the Spiro group [1] has identified the deoxy-sugar L-fucose (6-deoxy-L-galactose) as positioned on the non-reducing termini of a number of oligosaccharide chains of thyroglobulin. They and others suggest that this sugar is affixed to the glycoprotein in a final stage of its synthesis [1—3]. How L-fucose is supplied and used in the thyroid is less completely understood. A preliminary study of various rabbit organs, but not thyroid tissue, revealed that one route of biosynthesis of GDP-L-fucose in mammalian tissues is via the following pathway [4]: D-mannose 6-phosphate → D-mannose

This work is taken from a dissertation sumitted by W.L.R. to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the Ph.D. degree. Present address: McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706. Reprint request should be directed to G.S.S.

1-phosphate → GDP-D-mannose → GDP-4-keto-6-deoxy-D-mannose → GDP-L-fucose. The overall biosynthetic pathway from D-glucose to GDP-L-fucose is compatible with known biochemical reactions [4], and the conversion of GDP-D-mannose to GDP-L-fucose occurs by a mechanism common to the biosynthesis of 6-deoxyhexoses in plants, microorganisms, and mammals [5]. Evidence for the existence of this pathway in calf thyroid tissue was provided by Spiro and Spiro [6], who demonstrated the incorporation of label from D-[^{14}C]glucose into mannose, fucose, and other sugars of calf thyroglobulin. Other sources of L-fucose for thyroglobulin synthesis may be preformed fucose from dietary sources [7], or from degradation of thyroglobulin in the continual physiological process of release of the thyroid hormone [8]. In order to use preformed fucose derived from these sources an enzyme, fucokinase (ATP:6-deoxy-L-galactose 1-phosphotransferase, EC 2.7.1.52), would be necessary to permit phosphorylation of the sugar in the 1-position as a first stage in its conversion to GDP-L-fucose. Such an enzyme has been described in porcine liver [7,9], where dietary fucose might be expected to be present in significant amounts, and in porcine submaxillary gland [10], where rapid turnover of glycoprotein occurs. The existence of a fucokinase in the thyroid has been implied by three studies in which free radioactive L-fucose was incorporated into the carbohydrate of thyroid glycoproteins with no detectable incorporation of the fucose label into amino acids or other sugars [2,3,11]. We wish to report here on the existence and unique characteristics of a thyroid fucokinase which may play a significant role in the synthesis of thyroglobulin by the salvage of preformed fucose.

Materials and Methods

Reagents

L-Galactose and L-[^{14}C]galactose were generous gifts from Ms. P. Hebda and Dr. George Barber (Department of Biochemistry, The Ohio State University, Columbus, Ohio); α-L- and β-L-fucopyranosyl phosphates were generous gifts from Dr. H.S. Prihar and Dr. E.J. Behrman (Department of Biochemistry, The Ohio State University, Columbus, Ohio). All radioactive sugars and their derivatives with the exception of uniformly labeled L-[^{14}C]galactose were obtained from commercial sources. The scintillation solvent used in determining radioactivity in these experiments was that of Patterson and Greene [12].

Protein determination

Protein was estimated, with bovine serum albumin as the standard, by the sensitive biuret method [13], the Folin-phenol method [14], the Lowry method modified for use in the presence of sulfhydryl reagents [15], or by the A_{260nm}/A_{280nm} ratio technique based on the extinction coefficients for enolase and nucleic acid [16].

pH measurement

The pH of small aliquots of enzyme assay solution (20—50 μl) was measured at 37°C with an Ingold No. 14040 microelectrode (Instrumentation Laboratories, Lexington, Mass.), which had been standardized at 37°C and was kept in

37°C water between measurements. For the pH optimum studies with fucokinase, buffers were chosen that would not compete with ATP, would not chelate divalent magnesium ions, and would not complex L-fucose or other sugars [17]. For buffers to be used above pH 8, non-primary amine compounds were chosen to eliminate possible Schiff base formation between the reducing sugar and the primary amine [18]. Stock solutions of buffers were prepared so as to give buffered solutions of known constant ionic strength when diluted for use in enzyme assays [19,20].

Tissues

Frozen dog thyroids, which were purchased as a unit of two lobes from the same animal, were obtained from Pel-Freez Biologicals, Inc., Rogers, Arkansas. Thyroid tissue was shipped on solid CO_2 and stored at −20°C until needed. Just before use, the frozen tissue was thawed slowly in an appropriate buffer and cleaned as described for fresh tissue. To obtain fresh thyroid tissue, male mongrel dogs were killed with a stunning device followed by exsanguination. Thyroid lobes were excised immediately, wrapped in foil, and placed on ice. After no more than 20 min the surrounding fat, blood vessels, membranes, and parathyroids were dissected away on an iced watch glass. The cleaned thyroid lobes were then stored in the medium to be used in the succeeding experiment.

Fucokinase assay

Fucokinase activity was assayed by adapting a radiometric method, used similarly for the assay of nucleoside kinases [21], in which squares of anion-exchange paper retain radioactively-labeled L-fucose 1-phosphate product but allow unreacted radioactively labeled L-fucose substrate to be washed away. In the usual assay, 0.1 ml of enzyme solution and 0.2 ml of assay reagents were combined at 2°C in a 1.5 ml conical propylene tube with attached cap to provide final assay concentrations as follows: $MgSO_4$, 6 mM; ATP, 5 mM; KF, 8.33 mM; dithiothreitol, 3.33 mM; morpholinopropane sulfonic acid (MOPS), 90 mM, pH 6.46 at 37°C; L-[1-^{14}C]fucose (0.17 μCi/ml) 20 μM.

At appropriate times before or during incubation, 50-μl aliquots were transferred to 1.7 × 1.7 cm squares of SB-2 anion exchange paper (Reeve-Angel, Clifton, N.J.). Addition of samples to the squares was found to stop the enzyme-catalyzed reaction. Spotted squares, which had been prenumbered with pencil, were allowed to stand at least 20 min at room temperature before being washed with 3 changes of deionized water (30 mg/square per wash, 20 min/wash) to remove unreacted substrate. Damp square were drained and sorted as each was transferred to a glass scintillation vial. To avoid internal absorption of β-radiation by the paper, the labeled sugar phosphate was eluted from the anion exchange square by additon to each vial of 1.0 ml of a solution containing 0.2 M KCl and 0.1 M HCl followed by gentle shaking for 20 min. After elution, 10 ml of scintillation solvent was added for determination of radioactivity. A square, spotten before 37°C incubation was started, was always washed with the other squares, counted, and substracted as part of the background. If a total of 96 000 cpm was applied to the square, this "blank" value was about 500 cpm. The radioactivity of a spotted but unwashed square represents the total amount of substrate available and was used for determining the percent

conversion of L-fucose to L-fucose 1-phosphate during each sampling interval. Enzyme concentrations were chosen to catalyze product formation which was at least double the SB-2 square's background radioactivity after 20 min reaction and which did not exceed 5% conversion of substrate to product in 1 h. One unit of fucokinase activity is defined as the amount which is required to catalyze the formation of 1 nmol of L-fucose 1-phosphate per 20 min under the assay conditions described above.

Electrophoresis of enzyme preparations

Electrophoresis of fucokinase was carried out in 2% polyacrylamide gels containing 0.5% agarose and a phosphate buffer (0.1 M potassium phosphate, pH 7.93; 5 μM EDTA; 9.5% glycerol) by a modification of a procedure used by Peacock and Dungman [22]. Pre-electrophoresis, carried out with 0.1 M potassium phosphate/5 μM EDTA, pH 7.93, in the buffer compartments plus 5 mM sodium thioglycollate in the upper cathodic compartment of the electrophoresis chamber removed excess persulfate ion. A solution containing purified fucokinase (36 μl enzyme preparation, 10 μl glycerol, 4 μl 0.1% Bromphenol Blue) was applied just above the surface of the gel, and electrophoresis carried out for approximately 15 h with 0.05 M potassium phosphate, 2.5 μM EDTA, pH 7.44, in the anode and cathode buffer compartments and 5 mM sodium thioglycollate and 1 mM ATP in the cathode compartment. After electrophoresis, a third of the gel was cut off longitudinally, stained with Coomassie Blue, cleared, and optically scanned at 560 nm in a Gilford 240 spectrophotometer with a Model 2410 linear transport unit. In order to localize the fucokinase activity the unaltered, remaining 2/3 longitudinal section was cut into uniform 2 mm thick slices, each slice was placed on Parafilm and covered with 10 μl of an assay solution (18 mM $MgSO_4$; 15 mM ATP; 10 mM dithiothreitol; 19% glycerol; 271 mM MOPS, pH 6.46_{37}; 0.9 mM L-[1-^{14}C]fucose containing 0.043 μCi of radioactivity) and the droplet was covered with a square of Parafilm to prevent evaporation.

After 60 min at 23°C, 10 μl of 0.01 M dithiothreitol was added under the edge of the small Parafilm square and mixed with the main droplet. After an additional 60 min at 23°C, the Parafilm square was replaced by a 1 × 1 cm SB-2 square. After 20 min at 23°C, the SB-2 square with adhering gel was overturned and the gel slice was washed with 20 μl of water. After 15 min, the gels was overturned on the stationary SB-2 square and after a few seconds the gel was removed with a thin spatula and discarded. From this point, SB-2 squares were washed as in the assay for fucokinase activity.

Purification of fucokinase

Although a number of lots of enzyme were prepared, the following procedures present a typical case.

Preparation of crude extract and high-speed supernatants. 4 frozen dog thyroid lobes were thawed at 4°C in 1.03 M glycerol/0.005 M β-mercaptoethanol/5 μM EDTA/0.02 M MOPS, pH 7.80 at 0°C (Buffer 1) and cleaned as described above. The thyroid tisse (8.05 g) was minced with scissors and homogeneized with a Potter-Elvehjem homogenizer in 2 vols. Buffer 1. The crude homogenate was diluted to 75 ml with the same buffer, 0.2 ml was

stored at 2°C for fucokinase and protein assays, and the remainder was centrifuged for 15 min at 18 000 × *g*. The precipitate was discarded, the supernatant solution was again centrifuged for 15 min at 18 000 × *g*, and the pellet was discarded. The volume of the 18 000 × *g* supernatant was measured (50 ml), 0.21 ml was stored at 2°C for fucokinase and protein assays, and the remainder was centrifuged for 60 min at 179 000 × *g*. The volume of supernatant solution was measured (48 ml) and 0.5 ml was stored at 2°C for fucokinase and protein assays.

DEAE-cellulose chromatography. DEAE-cellulose was cycled in the presence of EDTA [23] before use. The high speed supernatant (47.5 ml) was applied to a 60-ml bed volume (1.5 × 34 cm) DEAE-cellulose column pre-equilibrated with Buffer 1. The column was eluted (5-ml fractions) as follows: Buffer 1 until 39 fractions had been collected; 0—0.15 M linear KCl concentration gradient in 150 ml Buffer 1; 200-ml wash with 0.15 M KCl in Buffer 1; 0.15—0.60 M linear KCl concentration gradient in 400 ml of Buffer 1. The fractions which contained fucokinase activity (625—680 ml) were combined and an aliquot (10 ml) was stored at 2°C for determination of fucokinase molecular weight by Sepharose 6B gel filtration. The remainder (50 ml) was dialyzed (28 h, 4°C) against three 500-ml changes of Buffer 1. After dialysis, the volume of the enzyme preparation was 52.8 ml.

Hydroxyapatite chromatography. An aliquot (12 ml, representing 1.54 g thyroid tissue) of the dialyzed DEAE-cellulose-fractionated fucokinase preparation was applied to a 4-ml bed volume (0.9 × 6.3 cm), hydroxyapatite (Bio Gel HT) column. The column was eluted with Buffer 1 until thirty-four 2-ml fractions had been collected. The column was then sequentially eluted by 40 ml of 0.005 M phosphate, 40 ml of 0.015 M phosphate and 20 ml of 0.05 M phosphate. These buffers of increasing phosphate concentration were prepared by combining appropriate amounts of Buffer 1 with 1.03 M glycerol 0.005 M β-mercaptoethanol/5 μM EDTA/0.1 M sodium potassium phosphate, pH 8.00 at 0°C. The fractions having fucokinase activity (79—84 ml) were combined. The purification table, to be presented subsequently, represents the purification of the 1.54 g thyroid tissue carried through to the hydroxyapatite fractionation step.

Results

Characterization of β-L-fucose 1-phosphate

Both crude homogenates and fractinated proteins from thyroid tissues were examined with respect to their action on fucose in the presence of ATP and Mg^{2+}. Crude homogenates yielded three separately detectable radioactive components when subjected to Bio Gel P-2 chromatography. The major and lightest component was unchanged as measured by its lack of absorption to SB-2 ion exchange paper and lack of migration in an electric field and was shown to be unchanged fucose through chromatographic comparison with standard fucose. The two remaining radioactive components appeared to be negatively charged by virtue of their absorption to SB-2 ion exchange paper. The heaviest of these two components was thought to be a nucleotide sugar but was not characterized further since it does not appear as a product from thyroid enzyme frac-

tions which have been treated to remove small molecules or fractionated by the purification schemes presented in this paper. The remaining component was suspected to be fucose phosphate and was characterized by an unequivocal procedure. The suspected fucose phosphate was isolated by paper electrophoresis where it traveled a distance equivalent to that traversed by authentic α- or β-L-fucose 1-phosphate. The association of the phosphate residue with the 1-position of fucose was verified by an examination of the lability of that residue and by the behavior of the suspected 1-phosphate to reducing reagents. Ishihara et al. [7], and Trujillo and Gan [11], demonstrated that fucose 1-phosphate is quite susceptible to acid hydrolysis as are most aldose 1-phosphates. Trujillo and Gan [11] showed complete conversion of fucose 1-phosphate to free sugar and phosphate by a 10-min exposure to 0.1 M HCl at 100°C. Our unknown behaved similarly under these conditions as evidenced by paper chromatography of the hydrolysis product against appropriate standards. Treatment of the suspected fucose 1-phosphate with sodium borohydride before and after acid hydrolysis followed by paper chromatography of the products provided the information that unhydrolyzed fucose phosphate was not reduced to L-fucitol whereas the hydrolyzed fucose phosphate was. Thus the phosphate group is masking the aldehyde function on the C_1 position.

These data confirm the nature of the sugar phosphate as L-fucose 1-phosphase but do not define the anomeric configuration. This last fact was established through the use of a paper electrophoresis system recently developed by Prihar and Behrman [24] which separates anomeric pairs of glycosyl-phosphates. The unknown fucose 1-phosphate traveled at the same R_F in this system as authentic β-L-fucose 1-phosphate and was clearly separated from α-L-fucose 1-phosphate. These data clearly confirm the product of the thyroid enzyme action as the β-anomer.

Assay validity

The fucokinase assay was found to be linear with respect to time for at least 60 min at 2 different temperatures (30 and 37°C) and in the pH range 6.3—8.6. At extremes of pH, the assay became nonlinear within 20 min. The fucokinase assay was linear with respect to the volume of purified enzyme assayed but did not become linear for crude preparations until the low molecular weight endogenous components had been removed by gel filtration.

In crude or purified fucokinase preparations, ATP and $MgSO_4$ were necessary components for the phosphorylation of radioactive L-fucose. In a crude extract, KF (8.33 mM), ovalbumin (5.83 mg/ml), and Triton X-100 (6.25 mg/ml) addition stimulated fucokinase to a small extent (~10%) probably by solubilizing the enzyme, by stabilizing the enzyme, or by stabilizing the product formed. In assays with fucokinase preparations of higher purity, ovalbumin and KF had no effect while deletion of the sulfhydryl reagent from the enzyme preparation and the assay solution caused rapid decay of fucokinase activity.

Since fucokinase was sometimes assayed at various ionic strengths and pH values the effects of these parameters on the retention of L-[^{14}C]fucose 1-phosphate by SB-2 anion exchange resin loaded paper was tested. Upon increasing the ionic strength of aliquots spotted on the squares, the percentage retention begins to drop slowly above 0.2 M KCl and reaches an 83% retention plateau in

the 0.75—1.0 MKCl concentration region. The retention of ^{14}C-labeled L-fucose 1-phosphate by SB-2 squares is the same throughout the pH range 5—10.5 but drops to 85% retention by pH 4.2. For most assays, tritiated L-fucose was unsuitable for assaying fucokinase because it produced high blank values.

Localization, stability and purification of fucokinase

The subcellular distribution of fucokinase in tissue extracts was determined by analysis of homogenates for activity and after centrifugation at 179 000 × *g* for 60 min. 98% of the initially measurable fucokinase was present in the 179 000 × *g* supernatant solution.

Although fucokinase appears to be stable in frozen thryoid tissue or in primary tissue extracts, once some of the endogenous tissue components are separated from the enzyme it loses activity slowly at 0—37°C and very rapidly when frozen. Consequently a number of reagents were tested, with Bio Gel P-2 filtered crude fucokinase extracts, for their abilities to stabilize fucokinase activity. Compounds tested included dithiothreitol, β-mercaptoethanol, sucrose, glycerol, Mg^{2+}, KCl, ovalbumin, and EDTA. Only the sulfhydryl reagents in conjunction with sucrose or glycerol proved effective in stabilizing the enzyme, permitting freezing of these preparations for periods up to a week with no detectably loss of activity. As fucokinase purity was increased, the enzyme proved to be most stable when held at 2—4°C in the presence of glycerol and a sulfhydryl reagent.

The addition of a low concentration of EDTA to purification buffers appears to improve the recovery and specific activity of fucokinase preparations, perhaps by protecting the enzyme from inactivation by heavy metals which are contained in the matrix of DEAE-cellulose (Peterson [22], 1970).

A comparison of the use of a linear KCl gradient or a discontinuous linear KCl gradient for DEAE-cellulose column elution may be made through observation of the elution patterns of Figs. 1 and 2. When a linear KCl concentration gradient was used for the elution of fucokinase from the ion exchange column (Fig. 1), the enzyme emerged from the column within a band of other protein as though the position of fucokinase in the elution pattern had been influenced by a mass effect of the charged groups of the other proteins being simultaneously eluted. The discontinuous linear gradient protocols, however, allowed the clearance of a large quantity of extraneous protein from the column before the elution of fucokinase occurred. As a consequence higher purifications were achieved.

A comparison of the use of linear and discontinuous hydroxylapatite column elution can be made by examining Figs. 3 and 4. More discrete separation of enzyme is seen to occur with discontinuous elution. The ionic strength of monovalent salts was not of concern in these protocols because fucokinase could not be eluted from hydroxyapatite by KCL concentrations as high as 1.0 M.

The dog thyroid fucokinase preparation with the highest specific activity was obtained by a combination of differential centrifugation. DEAE-cellulose chromatography, and hydroxyapatite chromatography as presented in Table I. If the percent recovery and the purification factor are calculated on the basis of the total number of fucokinase units present in the crude homogenate then

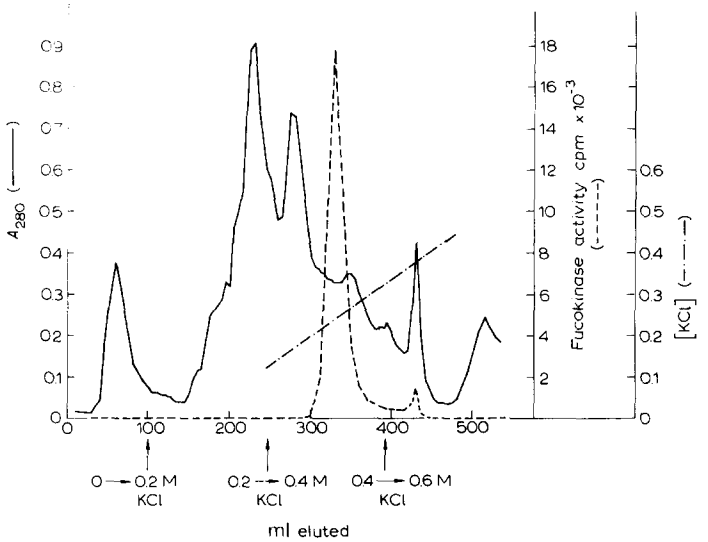

Fig. 1. Adsorption of a caninine thyroid fucokinase preparation to DEAE-cellulose with elution by a linear KCl gradient. Fucokinase activity is expressed as cpm/0.017 ml of the fraction incubated for 240 min. The preparation applied to this column had been purified by differential centrifugation and Bio Gel P-2 filtration.

the recovery is 31% and the purification factor is 2754 as shown in Table I. It should be noted, however, that a 2.53-fold increase in the apparent number of units of fucokinase occurred as the enzyme preparation was purified by centrifugation. If the recovery and purification are recalculated with the rationale that the number of fucokinase units in the 179 000 $\times g$ supernatant solution is representative of the total number of units present in the uninhibited crude

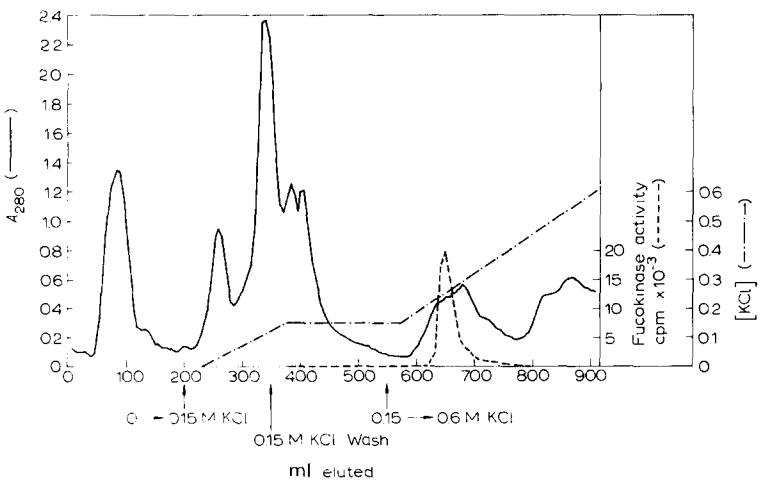

Fig. 2. Adsorption of a canine thyroid fucokinase preparation to DEAE-cellulose with elution by discontinuous and linear KCl gradients. Fucokinase activity is expressed as cpm/0.017 ml of the fraction incubated for 60 min. The preparation applied to the column had been purified by differential centrifugation.

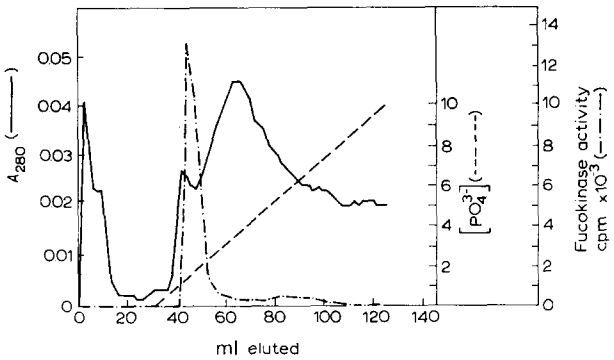

Fig. 3. Adsorption of a canine fucokinase preparation to a hydroxyapatite column with elution by a linear phosphate gradient. Fucokinase activity is expressed as cpm/0.017 ml of the fraction incubated for 2 h. The preparation applied to the column had been purified by differential centrifugation, by absorption to a DEAE-cellulose column with elution by discontinuous and linear KCl gradients, and by dialysis.

homogenate then the best overall purification and recovery should be divided by 2.53 to give purification factor of 1090 with 12.3% recovery. In other purification protocols where passage of a 179 000 $\times g$ supernatant solution through a Bio Gel P-6 column or a Bio Gel P-2 column was used to separate fucokinase from endogenous components of low molecular weight, an addi-

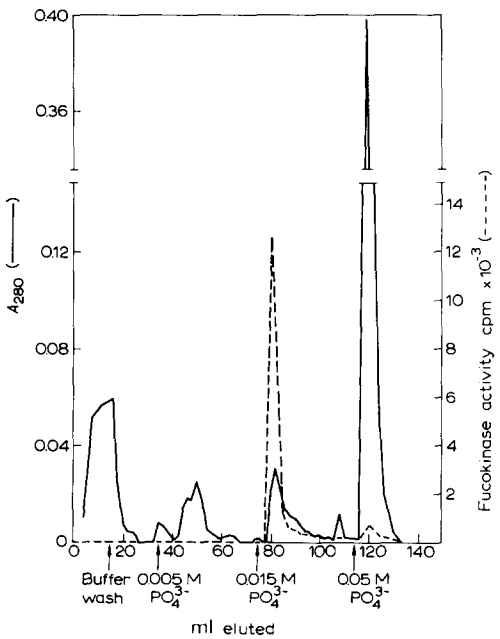

Fig. 4. Adsorption of a canine thyroid fucokinse preparation to a hydroxyapatite column with elution by a stepwise phosphate concentration gradient. Fucokinase activity is expressed as cpm/0.017 ml of the fraction. The preparation applied to the column had been purified by differential centrifugation, by adsorption to a DEAE-cellulose column with elution by discontinuous and linear KCl gradients, and by dialysis.

TABLE I

DOG THYROID FUCOKINASE PURIFICATION SCHEME

Fraction	Total units	Units/mg protein	Percent recovery	Purification factor
Crude homogenate *	22.2	0.066	100	1
18 000 × *g* supernatant solution	36.3	0.393	164	6
179 000 × *g* supernatant solution	56.2	1.06	253	16
Dialyzed combinated DEAE-cellulose fractions	43.6	8.05	196	122
Combined hydroxyapatite Fractions 80—84 ml	12.7	92.2	57	1397
Hydroxyapatite fractions 80 ml	6.92	182.1	31	2759

* Prepared from 1.54 g frozen dog thyroid tissue.

tional increase in the total number of fucokinase units occurred but the magnitude of this increase depended upon the dilution of the 179 000 × *g* supernatant solution prior to gel filtration. It is probably, then, that an endogenous thyroid tissue component of low molecular weight (<1800) can act as an inhibitor of dog thyroid fucokinase. When the purification procedure of Table I was completed in the shortest possible time the resulting fucokinase preparation had a specific activity of 126 units/mg protein and a 2.3-fold greater recovery than was observed in Table I.

Fig. 5 illustrates the results of agarose/polyacrylamide gel electrophoresis (Materials and Methods) of the combined fucokinase-containing fractions of Fig. 2. If all the protein in the shaded area is fucokinase, then fucokinase is approximately 39% of the total protein that passed into the gel.

pH optimum

A pH optimum curve was developed for the Sepharose 6B fucokinase preparation using acetate buffer for the pH region 4—6, imidazole buffer for the

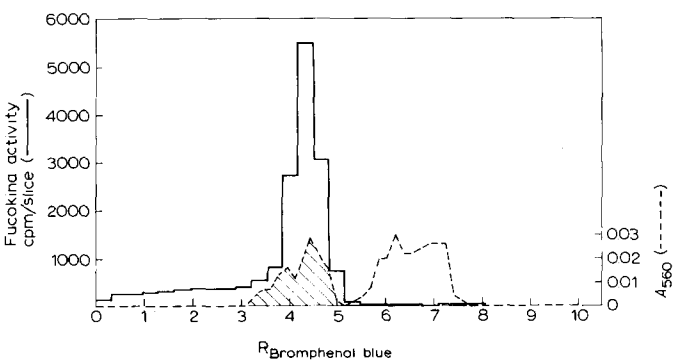

Fig. 5. Agarose-polyacrylamide gel electrophoresis of a Bio Gel HT-purified preparation from canine thyroids.

pH region 6—6.5, MOPS for the pH region 6 to 7.5, TAPS for the pH region 7—8.5 and triethylamine for the pH region 8.5—10. The pH optimum curve was biphasic with a lower plateau at pH 6.5 and a higher plateau at pH 8.3. The position of these plateaus was the same in less pure preparations that contained no endogenous components of low molecular weight.

Molecular weight

The molecular weight of fucokinase was determined by means of a Sepharose 6B column standardized with proteins of known molecular weight. When K_{av} values were calculated for the fucokinase activity peaks of 4 different preparations fractionated by gel filtration, it could be estimated that the molecular weight of dog thyroid fucokinase was 494 0000 ± 12 400 (S.E.M.). The molecular weight was independent of the degree of fucokinase purification prior to gel filtration.

Substrate specificity

Fucokinase derived from the hydroxyapatite step of Table I exhibited the following sugar specificities relative to L-fucose (1.00) at pH 8.3: D-glucose 9.21; D-mannose 8.69; 2-deoxy-D-glucose 5.76; *N*-acetyl-D-glucosamine 0.23; D-galactose 0.05; L-galactose 0.01; D-ribose 0.05; D-xylose 0.01. In crude homogenates, hexokinase activity had been 300-fold greater than fucokinase activity (as judged by phosphorylation of glucose) but in this stage of purity hexokinase activity was only 6- to 9-fold greater than fucokinase activity. When the negatively charged species formed from L-fucose, D-mannose, 2-deoxy-D-glucose, and D-glucose were subjected to mild acid hydrolysis (pH 1.3 for 7 min at 100°C) under conditions in which the phosphate group of *C*-1-phosphorylated sugars is known to be extremely labile to acid hydrolysis while phosphate groups found on other sugar carbons are nearly completely stable [26], it was found that the products formed from D-mannose, D-glucose, and 2-deoxy-D-glucose were negligible or only slightly hydrolyzed while the product formed from L-fucose was 95% hydrolyzed. This suggests that L-fucose was phosphorylated at C-1 by a unique enzyme and that the other sugars had been phosphorylated at carbons other than C-1.

If the combined hydroxyapatite fractions were further purified by gel filtration on Sepharose 6B (column described for mol. wt. estimation), hexokinase was separated from fucokinase (Fig. 6) and the resultant fucokinase preparation exhibited the following surgar specificities relative to L-fucose (1.00): D-ribose, 0.66; 2-deoxy-D-glucose, 0.35; D-galactose, 0.14; D-mannose, 0.04; D-glucose, 0.03 (each sugar was 41.67 μM). Thus Sepharose 6B does effect further purification of the fucokinase by removal of a major part of the extraneous "kinase" activities. This last procedure was not suitable as a routine purification step, however, because partial denaturation of the fucokinase activity occurred while the preparation was on the Sepharose 6B column. Thus denatured fucokinase eluted with the active enzyme and lowered the specific activity of the preparation.

Since with even the Sepharose 6B preparation some appreciable activity existed toward D-ribose and 2-deoxy-D-glucose an alternate approach was taken to evaluate substrate specificity. If ribose, for example, is an effective substrate

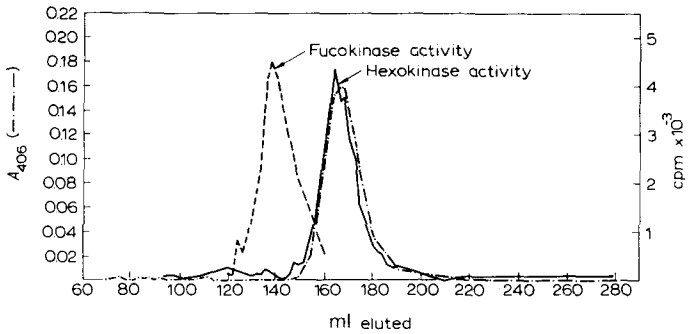

Fig. 6. Sepharose 6B gel filtration as a means of separation of fucokinase from hexokinase in a Bio Gel HT-purified preparation from canine thyroids. The *A* at 406 nm represents the marker beef liver catalase. Fucokinase activity is expressed as cpm/0.017 ml of the eluted enzyme fractions incubated for 20.8 h and hexokinase activity is expressed as cpm/0.017 ml of the eluted enzyme fractions incubated for 1 h.

for fucokinase it ought to compete for enzyme site and inhibit the enzyme's phosphorylation of radioactive fucose. If the ribose is serving as a substrate for some extraneous kinase impurity and not for fucokinase then no inhibition of fucokinase phosphorylation of labeled fucose should occur. Using this approach 8 non-radioactive sugars were tested for their inhibitory effect on the Sepharose 6B fucokinase preparation. As seen in Table II, none of the non-radioactive sugars appeared to inhibit the interaction of radioactive L-fucose with the active site of fucokinase. Hence none of these sugars are substrates for fucokinase. Thus it appears likely that fucokinase is highly specific for L-fucose.

The specificity of the enzyme for a phosphate donor was also examined. Of the following nucleotides studied only ATP exhibited phosphate donor capacity: ATP; ADP; AMP; GTP; GDP; GMP; ITP; TTP; CTP; UTP.

The metal ion requirements of fucokinase were examined replacing $MgSO_4$

TABLE II

EFFECT OF ADDED NON-RADIOACTIVE MONOSACCHARIDES UPON THE DOG THYROID FUCOKINASE-CATALYZED REACTON BETWEEN ATP AND L-[^{14}C]FUCOSE

Added unlabeled monosaccharide *	Relative fucokinase activity	
	pH 6.46_{37} **	pH 8.25_{37} ***
None	100	100
D-galactose	107	100
L-galactose	103	83
L-rhamnose	100	98
D-arabinose	105	102
D-glucose	101	95
D-mannose	96	94
D-ribose	101	100
2-deoxy-D-glucose	102	99
L-fucose	39	33

* Assay solution component concentration were: radioactive L-fucose, 30 μM; added monosaccharide, 60 μM.

** MOPS buffer.

*** TAPS buffer.

by $MnSO_4$, $CoSO_4$, $CaCl_2$, $FeSO_4$, $NiCl_2$, $ZnSO_4$, $SrCl_2$, $CdSO_4$, $BaCl_2$, $CuSO_4$ and NaCl. $MnSO_4$ exhibited 90% of the activity of $MgSO_4$ and $CoSO_4$ exhibited 12% of the activity of $MgSO_4$ in the fucokinase assay. The other ions were essentially ineffective.

Reciprocal velocity vs. reciprocal fucose concentration curves were plotted for the Sepharose 6B fucokinase preparation. A slight curvature away from linearity at high substrate concentration suggests an inhibitory effect of high levels of substrate [27]. Extrapolation of the curves to the 1/[s] axis gave apparent K_m values for L-fucose of 17 μM and 26 μM at pH 6.5 (MOPS buffer) and pH 8.3 (TAPS buffer) respectively.

The effect of variable ATP concentration on fucokinase activity was also examined at two pH values with Michaelis-Menten plots. Since the dissociation constant of $Mg \cdot ATP^{2-}$ is approximately 14 μM at pH 8 [27], nearly all the added ATP was maintained as the magnesium complex by adding a fixed 1-mM excess of magnesium over ATP. This 1-mM excess of free magnesium is rarely inhibitory and is probably close to the in vivo level [27]. Under these conditions, the concentration of the magnesium complex will be slightly lower at pH 6.46 than at pH 8.25. K_m values for ATP, estimated from double reciprocal plots of the Michaelis-Menten data, were 0.63 mM at pH 8.3 and 0.29 mM at pH 6.5.

Discussion

Two reports on procine liver fucokinase [7,9] and this report on canine thyroid fucokinase are the only studies in which the characteristics of the enzymes are delineated. A report of the isolation of a fucokinase from porcine salivary glands [10] is the only other report of a mammalian enzyme and few details concerning its characteristics are given.

It is to be anticipated that a canine thyroid fucokinase and a porcine liver fucokinase would be different entities as a consequence of both species and tissue differences. This report supports that supposition. Thus while pig liver fucokinase was just within the inclusion range of Sephadex G-100 [9] implying a molecular weight of approximately 150 000, the canine thyroid enzyme was found to have a molecular weight of 494 000 by Sepharose 6B gel filtration techniques. While the K_m of the liver enzyme has been reported as $1.2 \cdot 10^{-4}$ M [7], and $1 \cdot 10^{-4}$ M [9] for fucose and pH 8.0 and pH 7.3, respectively, the K_m for the thyroid enzyme was determined as $2.6 \cdot 10^{-5}$ M and $1.7 \cdot 10^{-5}$ M at pH 8.25 and pH 6.46, respectively. Thus the K_m for the thyroid fucokinase appears to be approximately one order of magnitude lower than that for the liver enzyme. The K_m for thyroid fucokinase with respect to ATP ($6.3 \cdot 10^{-4}$ M at pH 8.25) appears lower than for the liver enzyme ($2.9 \cdot 10^{-3}$ M at pH 7.3). However, comparison of these values are difficult since Yurchenco and Atkinson [9] used an invariant concentration of Mg^{2+} with varying concentrations of ATP and thus limited the amount of $Mg \cdot ATP^{2-}$ that could be formed. Uncomplexed ATP is normally inactive as a substrate with kinases and will, in fact, behave as an inhibitor if absorbed on the enzyme [27,28]. Thus the K_m for ATP for liver enzyme may actually be considerably higher than that reported by Yurchenco and Atkinson.

A comparison of the purification schemes for the canine thyroid and porcine liver enzymes also suggests that they are significantly different. Earlier attempts to purify the thyroid enzyme using ammonium sulphate precipitation, according to the procedure of Ishihara and Heath [7] were completely unsuccessful. Although the liver enzyme precipitates within a narrow range of ammonium sulphate concentration, the thyroid enzyme precipitated gradually over an extended range with no purification. The porcine liver enzyme elutes from DEAE-cellulose with a 0.1 M chloride ion concentration [9] whereas the dog thyroid enzyme eluted at 0.24 M chloride ion concentration. The porcine liver enzyme was absorbed to C_γ alumina gel in the presence of 0.03 M phosphate phate ion [7] while the thyroid enzyme was not. One must, of course, keep in mind that the differential absorption properties might vary with different absorbent preparations since the two enzymes have not been examined simultaneously with the same absorbent samples.

It is of interest that, with both the liver and thyroid fucokinase, an increase in the apparent number of enzyme units occurs in the early stages of purification as compared to the crude extract. Activation occurs as a results of high-speed centrifugation (Table I). Presumably particulate cell debris in some manner inhibits fucokinase activity. At present the nature of this inhibitory material is not known.

Substrate specificities are not readily used as an indication of differences in the enzymes from thyroid and liver. Working with the same enzyme from porcine liver Ishihara et al. [7] and Yurchenco and Atkinson [9] showed different specificities for the sugar substrate. The more recent work of Yurchenco and Atkinson shows extensive phosphorylation of D-glucose, D-galactose, and D-mannose as well as L-fucose by their purest enzyme preparation. The thyroid enzyme, at the hydroxyapatite step in the purification scheme, shows extensive phosphorylation of D-mannose and D-glucose as well. We have shown, however, that these "hexokinase-like" activities are lost by further treatment with Sepharose 6B. Thus we believe that thyroid fucokinase is a highly specific enzyme with respect to fucose and suspect that the variability of substrate specificity reported by other workers with the liver enzyme may well be due to enzyme impurities as suggested by Ishihara et al. [7]. It is possible that the reported acceptability of CTP, UTP, and GTP with the liver enzyme [7] is also a consequence of impurities in that preparation. The thyroid enzyme exhibited an absolute specificity for ATP.

The thyroid enzyme shows enzymatic activity over an extended range from pH 6.5 to 9.5. The pH of the cell milieu which immediately surrounds the fucokinase molecule is, of course, presently unknown. It is possible that it functions, however, both in the cytosol and the Golgi apparatus. Fucosyltransferase and other glycosyl transferase operate within the Golgi apparatus and exhibit pH optima in the range pH 5.2—6.5 [29]. Thus the extended fucokinase range may be a reflection of its use in several locales within the cell. It is of interest that the profile for guanosine disphosphate L-fucose pyrophosphorylase, the enzyme which takes L-fucose 1-phosphate (the product of fucokinase activity) to GDP-fucose (the substrate for fucosyltransferase), shows a remarkable similarity to that for fucokinase with the same extended pH range as fucokinase [30].

Ishihara et al. suggested that the enzyme fucokinase might be relatively widely distributed in mammalian tissues [7]. This report and that of Yurchenco and Atkinson [9] and Prohaska and Schenkel-Brunner [10] support that conjecture. It is indeed likely that any tissue which synthesizes a fucose-containing polysaccharide or glycoprotein contains a fucokinase. In most of these tissues this enzyme must serve to introduce dietary free fucose, derived from the blood stream into its appropriate metabolic scheme. Because of the extensive degradation and resynthesis of thyroglobulin in the thyroid gland we feel that thyroid fucokinase plays the additional role of a salvage enzyme to recycle fucose released from thyroglobulin.

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