Intra-Renal Distribution of Glucose-6-Phosphatase: Methodological and Physiological Considerations.* (31696)

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Previous reports indicate that glucose-6-phosphatase (G-6-P-ase) occurs in the proximal convolutions of the mammalian kidney, but its presence in other renal cortical structures is disputed(1,2,3). This leaves in doubt the identity of the renal cortical structures which effect gluconeogenesis, inasmuch as G-6-P-ase is a major clue to this.

The studies reported here were undertaken after considering possible causes of the discrepancies in earlier reports. Two of these (1,2) employed "analysis *in situ*" methods, the pitfalls of which have been discussed by Friedenwald(4). A third study(3) employed quantitative histochemical methods but histological sampling was controlled by an indirect "mapping" method(5,6) in which errors might have occurred.

Methods. Quantitative histochemical methods were used. Trypan blue was used as a supravital stain to distinguish directly between proximal and distal convolutions. This dye selectively stains the proximal convolutions except for the *pars recta*(7). Alkaline phosphatase was also studied as an additional check on histological sampling. This enzyme is confined mainly to the proximal convolutions(7). Two apparently healthy adult male mongrel dogs with normal serum creatinine concentrations were given trypan blue (20 mg/kg) intravenously. Forty-eight hours later and after withholding food but not water for 16 hours, the kidneys were rapidly removed under sodium pentobarbital anesthesia (30 mg/kg, intravenously) and were quickly cut in planes perpendicular to the capsule into blocks 2 to 3 mm thick. These blocks, representing all renal areas, were immediately frozen in liquid nitrogen. Lyophilized sections 20 μ thick were prepared by methods given previously(7). These were stored *in vacuo* at —20°C until dissected, when they were warmed to room temperature *in vacuo,* then brought into room air. The dissection, handling, weighing, and composition of the renal tissues so obtained have been described(7,8). Tissue samples of several types (Table I) ranging in dry weight from 0.3 to 1.0 μg were placed individually in the bottoms of small tubes(9) of 2 mm bore and 50 to 60 mm length. Enzyme activities were measured directly in these tubes by adding 3 μl aliquots of appropriate buffer-substrate mixtures (see below) to the dry samples, in an ice bath, followed by subsequent procedures as given below. Other general aspects of the microchemical procedures have been published(7, 8,9).

G-6-P-ase activities were measured by the method of Bonting *et al*(3) except that the incubation medium was altered slightly and contained the following reagents at the final concentrations specified: glucose-6-phosphate, 0.040 M; citrate buffer 0.02 M, adjusted to pH 6.35 with NaOH; disodium ethlenediaminetetraacetic acid (EDTA), 0.001 M; bovine serum albumin, 0.05%. The addition of EDTA improves the stability of the enzyme during incubation(10). The method was evaluated in this laboratory using aliquots of homogenates of dog kidney prepared in 0.25 M sucrose, added to aliquots of buffer-substrate mixtures which were prepared to give the desired reagent concentrations in the incubation mixtures. Optimal substrate and hydrogen ion concentrations were determined. The reaction conditions given above were found to provide maximal observed enzyme activities, linearly related to tissue content, and the K_m of the dog kidney enzyme observed (4.8×10^{-3} M) is similar to that of 4.0×10^{-3} M given by Bonting *et al*(3) for the human kidney enzyme.

Alkaline phosphatase activities were measured by a method previously studied and described in detail(7).

Results. The results are given in Table I. The data obtained from each dog were very similar and are pooled for convenience.

G-6-P-ase was found in the proximal (try-

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TABLE I. Distribution of Glucose-6-Phosphatase and Alkaline Phosphatase in Dog Kidney (Mean Activity* ± Standard Error).

Renal area	Glucose-6-phosphatase	Alkaline phosphatase
Prox	2.6 ± .19 (26)	14.6 ±1.12 (26)
Dist	2.4 ± .12 (27)	2.6 ± .29 (23)
Med ray	4.2 ± .33 (27)	.8 ± .19 (25)
Outer med	.3 ± .06 (15)	.5 ± .09 (13)
Inner med	.3 ± .09 (17)	<.1 ± .02 (16)
Papilla	.2 ± .06 (14)	<.1 ± .10 (10)

* Activity is expressed as moles of substrate hydrolyzed per kg of dry tissue per hr. Representative values for dry wt per liter of these renal areas have been given(7). Figures in parentheses = No. of separate tissue samples studied. Abbreviations: Prox = proximal convolutions; Dist = distal convolutions; Med ray = medullary ray; Outer med = outer medulla; Inner med = inner medulla.

pan blue stained) convolutions, the distal (unstained) convolutions, and the medullary rays, with much lower activities in the subcortical areas. The results agree with those of Bonting *et al*(3) in contrast to those of others (1,2). The relatively high activities in the medullary rays were also observed by Bonting *et al*(3) but no immediate explanation is apparent. These areas are a mixture of *pars recta,* loops of Henle and collecting ducts(7). By inference, since the subcortical areas containing loops and collecting ducts have low G-6-P-ase activities, some other element in the medullary rays must have high activities. It may be the *pars recta,* which also appears to be unusually rich in some other enzymes(7).

Alkaline phosphatase activities predominated greatly in the proximal convolutions, which agrees with previous findings(1,2,3,7) and indicates that good separations of proximal and distal convolutions were achieved. It is curious that "analysis *in situ*" methods appear to localize alkaline phosphatase activities accurately within differing renal tissue types, while the G-6-P-ase localization by these methods is inaccurate.

The present quantitative histochemical studies thus indicate that G-6-P-ase is quite widely distributed in the renal cortex. The results strengthen the bases for the recently proposed use of renal cortex for *in vitro* studies of gluconeogenesis(11) inasmuch as most of the renal cortical mass probably participates in this, and the mass of non-glucose producing tissue is correspondingly smaller than was apparent from the "analysis *in situ*" studies.

The low G-6-P-ase activities found in all subcortical areas indicate that these structures do not effect gluconeogenesis, and are compatible with the failure to demonstrate gluconeogenesis in fresh slices of renal medulla and papilla(12).

A previous discussion(12) of the role of gluconeogenesis in renal function, predicated in part on the assumption that gluconeogenesis occurs only in the proximal convolutions, must now be amended in view of these observations.

Summary. The intra-renal distribution of glucose-6-phosphatase was reinvestigated because of discrepancies in earlier reports on its presence in certain renal cortical structures, and because of its importance as an indicator of which renal cortical structures effect gluconeogenesis. Possible sources of error in previous histochemical studies were first considered, and quantitative histochemical methods were then chosen for the present studies, which avoid these problems. Glucose-6-phosphatase was found in the proximal and distal convoluted tubules and the medullary rays of the dog renal cortex. Its activities in all sub-cortical areas were very small in comparison to those in the cortical areas, which correlates well with other observations on gluconeogenesis in these tissues. Speculations on the role of gluconeogenesis in renal function must take these observations into account. Renal cortex also appears to be highly suitable tissue for *in vitro* studies of gluconeogenesis and factors which influence it.

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The Multiple Nature of Crystalline Egg-White Lysozyme. (31697)

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Early in the course of our studies on radiation effects in egg-white lysozyme(1,2), it became necessary to determine the purity of commercial crystalline preparations of the enzyme. Two or three times recrystallized material as supplied commercially is usually prepared by the method of Alderton and Fevold(3). However, this crystalline lysozyme has already been shown to be composed of at least 3 fractions by chromatography on columns of Amberlite XE-64(4), calcium phosphate columns(5) and carboxymethylcellulose columns(6).

Our work required gram amounts of homogeneous enzyme to permit the detection of small amounts of products arising from irradiation. Initial purification attempts involved passing solutions of the crystalline material through large preparative columns of BioRex 70 prepared and eluted essentially according to the procedure of Tallan and Stein(4). The 3 components previously recognized could be easily observed, but the shape of the elution curve for the main component and rechromatography experiments indicated homogeneity had not been obtained. Modification of the eluting buffer system and a slower rate of elution increased resolution to the point that as many as 7 components can now be demonstrated in crystalline lysozyme.

Materials and methods. Preparative columns measuring 6.0 × 75.0-80.0 cm were packed with BioRex 70, 200-400 mesh (Bio-Rad Laboratories). This resin is a weakly acidic carboxylic cation exchanger similar in nature to the XE-64 resin employed by Tallan and Stein(4). The resin had been previously placed in the hydrogen form by stirring 1 kg resin with 4 liters 3 *N* HCl overnight at room temperature. The resin was washed with distilled water until the filtrate was at pH 6 and then was suspended in 0.2 *M* potassium phosphate buffer, pH 7.09. The pH of the suspension was adjusted to pH 7.09 by the addition of 20% potassium hydroxide with continuous stirring. After filtering off the resin it was washed with 0.2 *M* potassium phosphate, pH 7.09, 10 liters per kg original resin weight. Fines were removed by suspending the resin in buffer (4 liters per kg), letting settle 20 minutes, and decanting the supernatant. This step was repeated until no cloudiness was visible in the decanted solution. Then, the resin was suspended in an equal volume of buffer and the slurry poured into the columns. The bottom of the column was fitted with coarse fritted glass disc on top of which was packed a half-inch layer of glass wool to prevent plugging of the disc during column operation.

Buffer was permitted to drain from the column under gravity pressure until the buffer surface was 1 to 2 inches above the top of the resin bed. Then, another aliquot of slurry was added and this step repeated until the desired column height was achieved. Buffer was passed through the column until the eluate emerging from the bottom was at pH 7.09. Then, 3.0 to 10.0 g crystalline egg-white lysozyme (Worthington Biochemical Corp. or Pentex, Inc.) was dissolved in buffer