Comparative Glycogenesis in the Liver and Glycogen Body of the Chick.* (28379)

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Differences between avian and mammalian carbohydrate metabolism are manifold and have received considerable attention. The resistance of birds to chemical and/or surgical diabetogenic techniques(1) as well as to administration of growth hormone(2) and insulin(2,3), yet the marked sensitivity of aves to glucagon(4,5) and certain sulfonylureas (4,6,7) document some of the observed differences in carbohydrate metabolism between this class and mammalia. Since the many parameters evaluated in the aforementioned reports involve the direct or indirect formation or hydrolysis of glycogen depots, a study of avian glycogen synthesis appeared desirable.

The purpose of the present study was to compare *in vivo* hepatic glycogenesis with that of the avian glycogen body, the latter tissue being a gelatinous structure in juxtaposition with the spinal cord, peculiar to aves, and containing a markedly higher glycogen concentration than other avian tissues (8). Preliminary studies (unpublished) indicated that the avian glycogen body failed to take up P^{32} within 24 hours after administration; however, spinal cord tissue taken from the same area readily took up the radioactive label. The observation that a 24-hour fast preceding feeding exaggerates glycogenic processes(9) was employed to evaluate the rate of glycogen synthesis from different C^{14}-hexoses by these 2 avian tissues. Furthermore, the reported consistency(8,10-12) of the carbohydrate moiety within the avian glycogen body merited investigation as to whether or not this structure used substrates other than glucose for its glycogen-precursor material.

Methods. All experiments were conducted on female New Hampshire chicks, 3-4 weeks old, which were fasted 20 hours prior to use. At the end of the fasting period each chick was tube-fed 15 ml of a gruel (0.5 g chick-growing mash/ml containing 21.4% protein, 67.0% carbohydrate and 7.0% fat) which was pipetted directly into the crop. One hour later this procedure was repeated with an identical quantity of gruel, 30 minutes after which a preisotope blood sample (0.5 ml) was drawn by cardiac puncture. Immediately thereafter the radioactively labeled hexose (either glucose, fructose or galactose) was injected intracardially in a volume of 0.2 ml containing 1-50 μc. Since all studies yielded nearly identical results, differing only in total radioactivity circulated and incorporated into glycogen, only those studies where 10.5 μc were injected/chick are reported here.

The C^{14}-labeled hexose§ was allowed to equilibrate *in vivo* for ½, 1, 2, 3, 4, 8 or 24 hours during which time the birds had access to water but not to food. In the glucose and fructose studies an additional 24-hour group of birds was allowed to eat *ad libitum* until time of sacrifice. At the end of the equilibration period, a terminal blood sample of 0.2 ml was obtained by cardiac puncture, the chicks narcotized, and liver slices (60-100 mg) placed in tared test tubes containing 30% KOH. The entire glycogen body was excised from its location dorsal to the sacral portion of the avian spinal cord, weighed to the nearest 0.1 mg and also placed in 30% KOH.

Blood glucoses were determined by Nelson's modification of the Somogyi method (13); tissue glycogens were analyzed in duplicate by the anthrone micro-method of Seifter *et al.*(14) and twice were precipitated with ethanol, dissolved in water and centri-

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§ Uniformly labeled glucose-C^{14} and fructose-C^{14} obtained from New England Nuclear Co., Boston; Galactose-1-C^{14} obtained from Dr. H. Isbell, National Bureau of Standards, Washington, D.C.

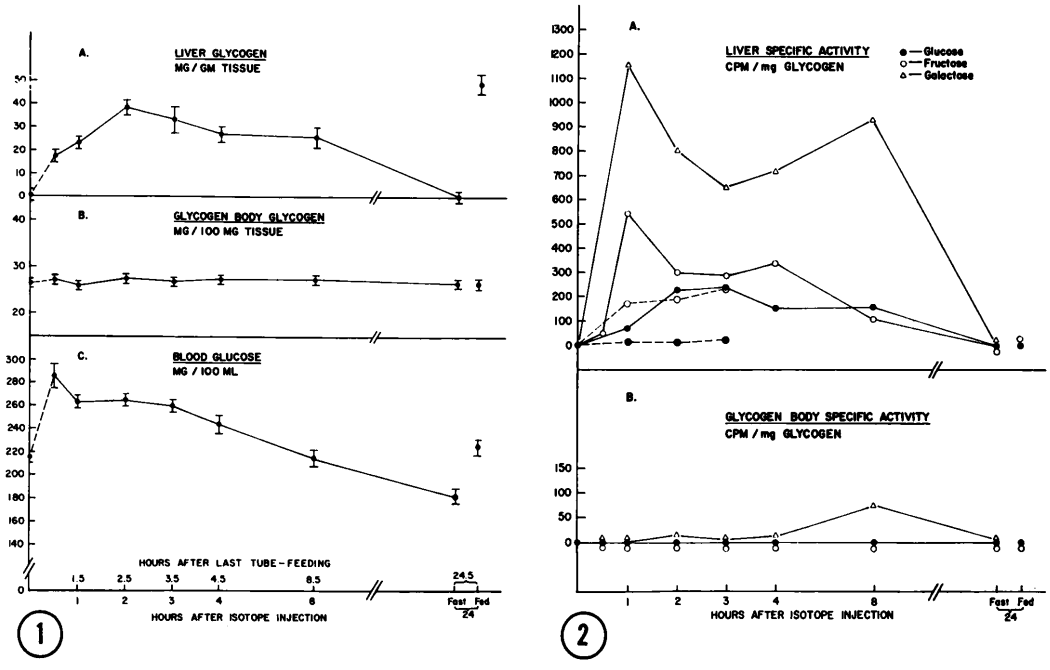

FIG. 1. Effect of tube-feeding a test meal on total liver glycogen, total glycogen body glycogen and blood glucose of previously fasted chicks. Each point represents the mean of 12 observations ± standard error (vertical bars) except for the 24-hr fed group. The latter data are the avg of 8 chicks. Time '0' is 20 hr after last *ad libitum* feeding; time '1' is 1½ hr after last tube-feeding, thus 1 hr after isotope injection.

FIG. 2. Comparison of incorporation of C-14 glucose, fructose, or galactose into liver or glycogen body glycogen. Feeding, timing and observation as in Fig. 1. Curves with broken lines (2A) indicate calculated theoretical indiscriminate uptake values using galactose as reference.

fuged before the final dissolving in 2 ml of water. One aliquot of this was diluted for glycogen analyses; another aliquot (0.5 ml) was plated for counting on a Nuclear-Chicago Model D-47 gas-flow counter. The final counts were expressed as specific activity (counts/min/mg total glycogen).

Results. The glycogenic and hyperglycemic effects of 2 tube-feedings (one hour apart) of chick mash in porridge form to fasting chicks is shown in Fig. 1. The first value given (zero time) denotes the concentrations at the end of a 20-hour fast, and thus immediately prior to the first feeding. The dotted lines indicate a period of 2 hours after the first tube-feeding and thus ½ hour after the isotope was injected. The rapid total hepatic glycogen accumulation was quite obvious and prominent (Fig. 1A), though these levels did not quite reach those of livers taken from *ad libitum* fed chicks. The rapid rise in blood glucose (Fig. 1C) was also quite evident, and reached a plateau between post-absorptive hours 1 and 3, only to decline subsequently. Concomitantly, liver glycogen concentrations continued to level off for an additional 5 hours before decreasing as a result of prolongation of the fasting period. In contrast to these excursions in hepatic glycogen and blood glucose levels, the avian glycogen body polysaccharide levels were not only unaffected by the preceding 20-hour fast but also remained virtually constant throughout the entire observation period during which early liver glycogenesis and subsequent glycogenolysis were very much in evidence (Fig. 1B). The standard error of the mean for these glycogen levels varied only 0.3-0.7 mg/100 mg glycogen body tissue, data which complement and document further the stability of the polysaccharide within this avian structure. These observations are in excellent accord with earlier reports(8,12,15).

A comparison of the avidity with which

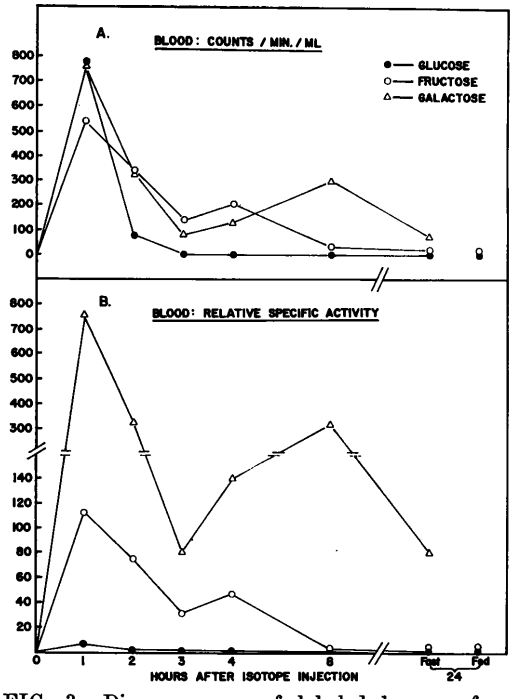

FIG. 3. Disappearance of labeled hexoses from blood of tube-fed chicks. Feeding, timing and observation as in Fig. 1. Relative specific activity calculated as CPM/ml blood/pool size based on concentration ratios of 200:5:1 for glucose:fructose:galactose, respectively.

both liver and glycogen body tissue took up the various labeled hexoses and incorporated them into glycogen is indicated in Fig. 2A and 2B. It is obvious that the avian glycogen body was not actively incorporating glucose, fructose or galactose under the conditions of the present study (Fig. 2B). Even though a small amount of galactose was found in the glycogen compartments of this structure 8 hours after the isotope was administered, the physiological significance of such a small and delayed incorporation is somewhat doubtful. In contrast to the glycogen body, the avian liver readily incorporated all 3 hexose labels within the polysaccharide (Fig. 2A). Though the rate and amounts of uptake of the radioactive label by the liver varied with the hexose involved, all 3 incorporations reached a plateau between 2-4 hours after isotope injection and declined to near vanishing levels by 24 hours. The persistence of isotope activity in the circulation after injection of fructose and galactose may be due to a sequestering and subsequent release of the hexoses, or due to labeled degradation products. Because of this possible appearance of labeled metabolites in the blood, as well as to possible recycling phenomena, calculations beyond the 3-hour point are probably meaningless. The relative specific activity of the sugars in blood using the calculated pool size ratios for circulating glucose:fructose:galactose (200:5:1; Hazelwood, unpublished) are plotted in Fig. 3B. These data, in conjunction with the calculated theoretical hepatic uptake curves of Fig. 2A, indicate that the rapid rise observed in hepatic glycogen label with galactose-C^{14}, and to a lesser extent with fructose-C^{14}, need not be interpreted as more rapid hepatic glycogenesis from these precursors but rather as much smaller circulating fructose and galactose pools. Thus, these 2 hexose-substrate pools are labeled considerably higher than that of the blood glucose pool upon reaching the liver cell and subsequently becoming incorporated within the glycogen molecule. Assuming indiscriminate uptake of the various sugars by the liver cell, the calculated theoretical curves in Fig. 2A (broken lines) using galactose-C^{14} glycogen as a reference lie considerably below the observed respective specific activity curves for fructose and glucose. It may be concluded, therefore, that glucose is used preferentially over fructose as a glycogenic substrate in avian liver, and that both of these hexoses are employed preferentially over galactose.

Discussion. Hepatic glycogenesis and glycogenolysis appear to be more rapid in the chick than in other laboratory animals (2, 16); however, data are sparse as to the rate of glycogen synthesis or turnover. Recently, it has been reported that training rats to eat their food within a restricted time period following a 24-hour fast exaggerates the glycogenic picture in many different tissues. Also, the peak of hepatic glycogen incorporation of glucose-C^{14} appeared to occur about 3 hours after commencement of eating; yet total glycogen deposition reached a peak in these rats at about the 7th post-absorptive hour(9). The data presented herein employing chicks are in good agreement with those

of the rat for liver tissue. By adding 1.5 hours (time from first tube-fed gruel to isotope injection) to the time along the axis in Fig. 1, the glycogenic peak occurs 3-4 hours later and total glycogen deposition lies somewhere between 4-8 hours later. Further evidence is lent to this similarity of avian glycogenesis with that of the rat by the observations of Weber *et al.*(16) who found that the depletion of liver carbohydrate enzyme concentration in chicks by starvation, and reappearance by refeeding, paralleled the alterations observed in rats under identical dietary regimens.

While the polysaccharide level of the avian glycogen body has been shown to increase greatly during embryonic growth(10,11), in the older bird it is very resistant to alteration after death(8), due to the dietary manipulation(15) or hormone administration (12). Thus, its stability and constancy present a physiological enigma to students of carbohydrate metabolism. Watterson *et al,* have demonstrated decreased glycogen levels in this structure if decapitate-hypophysectomy was performed early in embryonic life (17). Complementary data have been obtained in our laboratory with the use of avian pituitary extracts(12). In the present study, and under conditions where the avian liver was virtually depleted of glycogen (20-hour fast), or rapid glycogen formation (tube feedings) with subsequent slow "homeostatic" hydrolysis (post-absorptive period), the glycogen body glycogen concentration was not altered and the rate of radioactive hexose incorporation was negligible. While it has been demonstrated in the rat that hepatic glycogenesis from a specific hexose is favored most by a pre-feeding regimen employing that same hexose as the major dietary carbohydrate constituent(18), the glycogen body still failed to demonstrate active incorporation of the glucose-C^{14} label into glycogen after the high carbohydrate (starch) gruel was pipetted into the chick crop sac. These results are in excellent agreement with those indicative of glycogen body inactivity presented by DeGennaro (abstract) while this report was in preparation. He observed little disappearance of glycogen-C^{14} in the avian glycogen body one week after hatching if glucose-C^{14} was injected into fertile eggs on the 10th day of incubation(19). Thus, these data as well as those of the present study fail to elucidate the physiological function and/or importance of the avian glycogen body.

Summary. The incorporation of C^{14}-labeled hexoses into glycogen compartments of avian liver and glycogen body was studied in young chicks which had been fasted 20 hours and tube-fed a test gruel at ½ and at 1-1½ hours prior to isotopic administration. The results indicate that in contrast to rapid liver incorporation of the hexose label, the avian glycogen body was not active as far as glycogenesis was concerned for at least 24 hours after isotope injection. Only a trace of the galactose label was found in the glycogen body 8 hours later, no activity from glucose or fructose-C^{14} being found at any time in this structure throughout the 24-hour post-injection observation period.

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Effect of Difluoromalonic Acid Di-Amide on Oxidation of Organic Acids by *Pseudomonas aeruginosa.* (28380)

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There have apparently been no studies on the effects of fluoromalonates on bacteria. Chari-Bitron(1) describes the action of monofluoromalonate in animals. He showed that it was decarboxylated to fluoroacetate and thus caused citrate accumulation. The succinic dehydrogenase and oxalacetic decarboxylase were only slightly inhibited by the compound. The following is a study of the action of difluoromalonate on washed suspensions of a strain of *Pseudomonas aeruginosa.* The oxidation of a number of organic acids including succinate, fumarate, and malate was inhibited by the compound. But when the cells were broken no inhibition, even with high concentrations, was found. It would appear that difluoromalonate is a non-specific inhibitor of the transport of these acids. Monofluoromalonate is much less effective and tetrafluorosuccinate and hexafluoroglutarate are inactive in the concentrations used.

Experimental. Pseudomonas aeruginosa was grown for 24 hours at 34° in Bacto nutrient broth, centrifuged and washed twice with water. The cells were taken up in 14 ml of 0.05 M Na-K phosphate buffer pH 7.8 and 0.5 ml was used in each Warburg vessel which had a final fluid of 2.0 ml. The fluorinated compounds, obtained from the Hynes Chemical Co. of Durham, were used as the di-amides. Some experiments done with the sodium salt of difluoromalonic acid showed that it had the same effects as the amide. Cells were broken by placing them in a 10 KC Raytheon sonic vibrator in the cold for 2 minutes. Unbroken cells were centrifuged down and 0.75 ml of the supernate was added to a mixture of the substrate, 0.7×10^{-3} NaCN and 1.0 ml of 0.46×10^{-4} M sodium 2,6-dichloroindophenol in a final volume of 3.0 ml. The rate of dye reduction was measured in a spectrophotometer at 600 mμ.

Difluoromalonamide (DFMA) was added to the cells before the substrate or 60 minutes after the substrate when the oxidation rate was linear. As shown in Table I for succinate and fumarate the inhibition percentages obtained are the same for both conditions which indicates that DFMA does not specifically inhibit possible permease induction. The same is true for benzoate oxidation which involves enzyme induction. In both cases a 70% inhibition occurred. (The inhibition percentages are the average of 4 readings taken during the 60 minutes following a 30 minute initial period except when enzymes are induced. Then they are the mean for 4 readings taken when linear oxidation rate is obtained.) After the first hour the inhibition percentage decreases partly because the control oxidation rate falls off and partly because some deamidation of DFMA occurs and the ammonium ion stimulates the oxidation rate of most of the substrates.

The inhibition percentage by DFMA is in-

TABLE I. Effect of 100 μg/ml of DFMA Added to the Cells (A) 15 Minutes Before or (B) 60 Minutes After 500 μg/ml of Succinic or Fumaric Acid on Oxidation of these Substrates.

Substrate	A	B
Succinate	70	68
Fumarate	64	60