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IN VIVO AND IN VITRO STUDIES OF GLUCOSE METABOLISM IN THE CHICKEN

By

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ABSTRACT

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Glucose replacement rate, percent recycling, mean transit time, and glucose mass were examined using various double-labelled glucose tracers. Estimates of replacement rate were greatest for 2-^{3}H glucose (2T), with 3T, 4T, 5T and 6T glucose all having similar values. Calculated glucose mass based on all tritiated tracers agreed closely with the direct determination of body glucose (734-1086 mg/kg body weight vs. 969 mg/kg). Reincorporation of tritium from $^{3}H_2O$ into glucose did not occur to any significant degree. The young chick was found to have a rapid rate of glucose turnover and high percent recycling compared to mammals.

When chickens were fasted for 1, 4, or 8 days, significant decreases occurred in total body protein and fat with fasting, the greatest energy loss from fat. Glucose production remained remained constant with fasting at 10-13 mg/min/kg body weight. Blood lactate and glycerol were unchanged with fasting, while pyruvate increased and plateaued. Plasma alanine, serine and glycine levels were high compared to values in fasted mammals.

Blood β-hydroxybutyrate increased dramatically with fasting (350-3500 nmol/ml), while acetoacetate remained constant. The hepatic lactate:pyruvate ratio was unchanged with fasting, while the β-hydroxybutyrate:acetoacetate ratio increased. These ratios may influence phosphoenolpyruvate and glucose production in mammals. Hepatic and renal phosphoenolpyruvate carboxykinase levels remained constant, while hepatic lactate dehydrogenase increased with fasting. β-hydroxybutyrate dehydrogenase levels were very low at all times. The results indicated that little glucose sparing adaptation per kg occurs in the chicken with fasting.

Glucose production was studied in the isolated hepatocyte. The highest rate of glucose production was obtained from lactate, followed by dihydroxyacetone, glyceraldehyde and fructose. Alanine was converted to glucose at only 4% the rate of lactate, in contrast to the rat and guinea pig, where it is converted at 40-50% the rate of lactate. Pyruvate was utilized to form glucose at 20-30% the rate of lactate, again in contast to rat and guinea pig, where lactate and pyruvate are utilized equally well. Addition of 10mM sorbitol, xylitol, or ethanol increased glucose production from pyruvate 25-40%, while glycerol addition increased it only 9%. Addition of β-hydroxybutyrate had no effect on glucose production from lactate or pyruvate. Addition of octanoate had no effect on glucose production from pyruvate, but depressed it from lactate at 5mM. Differences in the formation of glucose from various subatrates suggest some basic differences in the mode of

glucose production between the chick, and the rat and guinea pig.

Lactate and alanine turnover and percent glucose arising from lactate and alanine were calculated in vivo. Lactate turnover was 12.1 umole/min/kg, while alanine turnover was 6.9 umole/min/kg. The percent of glucose derived from lactate was 47%, while that of alanine was 8.5%. The results also indicated a significant conversion of ^{14}C alanine to ^{14}C lactate. The estimate of alanine conversion to glucose in vivo is higher than in vitro and may indicate that organs other than the liver contribute to alanine conversion to glucose. Lactate conversion to glucose was higher than in other species.

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REVIEW OF LITERATURE

Introduction

The use of experimental animals to detail intermediary metabolism and its regulation is a generally accepted research technique to gain specific information which might be applicable to human metabolism. The laboratory rat is usually the animal of choice, for obvious reasons. However, it has become clear that the rat is not the "ideal" human model. The only truly ideal human model is the human himself. But with increasing restrictions on human experimentation, another approach may be valid; that is, to use a variety of species for experimentation, choosing a particular species for study based on how closely it resembles the human in that particular situation.

The chick may represent such a model when control and maintenance of blood glucose levels in humans is considered. Even though several features of avian carbohydrate metabolism appear applicable to various pathological human disorders, very little study of avian intermediary carbohydrate metabolism has occurred. Possessing blood glucose levels of 150-300 mg/100 ml, the chick is an excellent model for hyperglycemia. In addition, glucagon, not insulin is the primary blood glucose regulatory hormone, in contrast to mammals, but similar to diabetes where insulin exerts little effect on blood glucose levels. The chick also appears able to resist severe fasting hypoglycemia, a capability important in humans and sometimes lacking. Thus, the chick may serve as a model for abnormal human glucose metabolism, especially abnormalities

in glucose production.

The importance of an adequate ability to regulate glucose metabolism begins at birth in humans, when the infant's blood glucose supply from the mother is severed. From this point, the neonate's blood glucose level depends on a balance between his tissues' utilization, the availability of his liver glycogen, and his ability to make glucose from lactate, glycerol, and amino acids. The enzyme phosphoenolpyruvate carboxykinase (PEPCK), glucagon, and catecholamines are usually elevated at this time and the infant's blood glucose levels are maintained. If enzyme, substrates, or hormones' effects are not existent at this time, neonatal hypoglycemia occurs, with concomitant problems in nervous function. Thus, adequate maintenance of blood glucose is of utmost importance in proper development of the human neonate.

In contrast is the diabetic human with severe hyperglycemia. In these people, a 100 g glucose load may result in 50-75 g of glucose escaping into the peripheral circulation, while in normal subjects only 30-40 g of glucose escape hepatic uptake (Felig,1975). Therefore, postprandially, diabetics are found to have higher peripheral blood glucose levels. As the diabetes progresses, hyperglycemia also characterizes the fasting state. In this condition, the liver loses its sensitivity to rising blood glucose levels and continues to produce glucose. In normal subjects, rising blood glucose levels would signal the liver to decrease glucose output. Splanchnic uptake of glucose precursors

is also elevated in the fasted diabetic. This ensures that sufficient substrate is available to the liver for continued glucose production.

The consistently high blood glucose levels in the chick lead to speculation on the control of blood glucose in this species and perhaps to a partial answer to glucose overproduction in the human diabetic. Thus, it was of interest to examine avian gluccse metabolism in some detail. The research is divided into four areas: tracer methodology to measure glucose production, tracer methodology for measuring alanine and lactate conversion to glucose, the use of isolated hepatocytes , and the role of redox state.

Tracer Methodology for Glucose Turnover

Basic to the study of carbon flux in animals in vivo is the determination of glucose production (utilization) and turnover rate. Katz et al (1974 a,b) defined some important concepts in establishing methods of evaluation of glucose carbon flux. The labelled glucose tracer may be given by a single pulse or a continuous intravenous infusion, the mathematical treatment being slightly different in each case. The plasma sampling pool is assumed to equilibrate with the tracer immediately and may be treated as one of a series of pools or by non-compartmental analysis (Katz et al,1974 a,b). The organism is also assumed to be in a metabolic steady state (postabsorptive). In this case, the rate of disappearance of tracer glucose from the plasma (rate of phosphorylation throughout the body) is equal to the rate of

production (release by liver or kidney)(Katz et al, 1974a; 1974b).

Many studies have used ^{14}C glucose to estimate the above parameters. However, this label usually leads to an underestimate of glucose utilization because of the recycling of labelled carbon from tricarbon units or CO_2 back into glucose (Katz et al, 1974a; 1974b; Brockman et al, 1975; Shipley and Gibbon, 1975). Labelled ^{14}C-lactate from extrahepatic tissues and any ^{14}C-lactate derived from hepatic metabolism may be recycled to ^{14}C-glucose in the liver and released into the blood. Thus, the rate of disappearance of blood ^{14}C-glucose would be less than if no recycling occurred.

The search for an ideal label, which would not recycle back into glucose, resulted in the proposal of ^{3}H-glucose tracers (Katz and Dunn, 1967; Dunn et al, 1967). The ^{3}H is lost irreversibly to water during extrahepatic glucose catabolism (Katz et al, 1974a; 1974b).

Originally, all ^{3}H glucose tracers were considered appropriate except 1-^{3}H glucose (Katz et al, 1974a; 1974b). More recently, questions have arisen of the meaning of data derived with several of the ^{3}H tracers. The 2-^{3}H glucose is presumed to lose ^{3}H at the isomerization of glucose-6-phosphate and fructose-6-phosphate (Katz and Rognstad, 1976). Approximately 50% of ^{3}H in position 2 of glucose-6-phosphate and position 1 of fructose-6-phosphate is lost in each

isomerization (Katz and Rognstad,1976). The hexose isomerase has been reported to possess high enough activity to shuttle the compounds back and forth several times before further metabolism (Katz and Rognstad,1976). Immediately obvious is a problem with 2-^{3}H-glucose tracers. If glucose-6-phosphate can be rapidly converted to fructose-6-phosphate and vice versa, glucose-6-phosphate could then return to glucose, without retaining the ^{3}H label, since gluconeogenic flow will never be completely nil (Clark et al,1974). Thus, through "futile cycling" glucose will lose ^{3}H without metabolism to tricarbon units.

The label of 6-^{3}H-glucose is presumed lost from the methyl group of pyruvate in carboxylation to oxaloacetate. If oxaloacetate, malate, and fumarate equilibrate, the methyl label may be further diluted (Katz and Rognstad,1976). Since the ^{3}H loss occurs late in glucose metabolism, the 6-^{3}H-glucose has been proposed as truly indicative of glucose utilization (production). In most species, the rate of loss of 6-^{3}H is much lower than 2-^{3}H, and slightly lower than 3-^{3}H, 4-^{3}H, or 5-^{3}H. The former three labels are lost at the triose stage of metabolism (Katz and Rognstad,1976). Futile cycling is also possible with 5-^{3}H-glucose, due to an excess of aldolase and triose phosphate isomerase resulting in rapid ^{3}H loss from fructose-1,6-diphosphate (Clark et al,1974). However, this has been disputed by Rognstad et al (1975). They found significant retention of ^{3}H during fructose conversion to glucose via fructokinase (fructose is metabolized

by conversion to tricarbon units after phosphorylation, then converted to glucose, CO_2, or lactate).

Thus, even though all ^3H tracers should provide valid estimates of glucose production, 6-^{3}H-glucose has been found most satisfactory (Dunn et al,1976; Katz et al,1976; Anwer et al,1976; Belo et al,1976). Both 2-^3H and 5-^3H glucoses have been used for estimation of futile cycling at their respective stages of glycolysis. Bloxham et al (1973) and Clark et al (1973) have shown increased futile cycling in the flight muscle of bumblebees exposed to cold. They have postulated that futile cycles are a method of fine regulation of metabolism. The futile cycling in bumblebee muscle which produces heat has been found necessary to allow the muscle to warm enough to prepare for flight.

Thus, all tracers have potential use. The studies reported here make use of several of the methods mentioned above.

Lactate and Alanine Turnover

The rate of glucose appearance indicates the total glucose production from all substrates, but does not indicate the quantitative importance of the various glucose precursors. It is possible to measure not only glucose turnover, but also the turnover of key substrates related to glucose metabolism such as lactate and alanine. It is also possible to measure the interconversion of glucose with either lactate or alanine by several methods.

Although it is feasible to use a single pulse injection to measure lactate or alanine turnover, Forbath et al (1967)

compared this technique to that of constant infusion for the estimate of lactate turnover. The constant infusion method estimated lower rates of lactate production than obtained with the single injection technique. The authors attributed the discrepancy to a high estimate of the lactate pool and apparent distribution space. The value is derived from the intercept of the terminal portion of the specific activity versus time curve. They postulated that the problem inherent in the method was the metabolism of tracer before equilibration between a rapidly mixing pool (blood) and peripheral compartments. In a multicompartmental system, the overestimate of pool size by this technique occurs if the substrate turns over at a fast rate. The primed infusion estimates rates in the dynamic steady state without reference to the amount of tracer intermixing. However, it should be noted that the single injection can be used sucessfully. Belo et al (1977) found the rates of lactate, alanine, and serine turnover in dogs on various diets with the single injection method. The rates for lactate turnover were similar to those obtained by Forbath et al (1967) with the constant infusion technique.

The rates of interconversion of metabolites have been measured by several methods. Depocas and DeFreitas (1970) proposed a model for glucose-lactate or glucose-glycerol interconversion. Their model involves injecting tracer amounts of m_1 (glucose) and m_2 (lactate) into two different animals. According to their theory, the rate of total net formation

of each compound from all sources can be measured, as well as the rate that m_1 is formed from m_2 and the rate at which m_1 is formed from all other precursors. The same calculations can be made for m_2. Kreisberg et al (1970) used essentially the same technique, but injected each tracer into lean and obese subjects on successive days, rather than pairing subjects.

The method of sampling in metabolite turnover studies has also been shown to be of importance (Chiasson et al, 1977; Reilly and Chandrasena,1977). This includes differences between whole blood and plasma and also arterial vs. venous sampling. Chiasson et al (1977) discussed methodological approaches for in vivo tracer work. It was noted that specific activity of alanine in the plasma of fasting subjects differed greatly from that in whole blood following constant infusion. They estimated that 90% of the alanine extracted from the splanchnic bed was from the plasma compartment. This is an important distinction because it is most correct to use the actual specific activity to which the liver is exposed in the calculation of precursor conversion to glucose. However, this is difficult as the liver's blood supply is complicated, as dicussed below. Bergman and Heitman (1978) also found that plasma and red blood cell amino acid content did not rapidly equilibrate in sheep.

Reilly and Chandrasena (1977) assessed the errors in lactate turnover in sheep which resulted from sampling

jugular vein rather than carotid artery blood. The main problem in the sampling of the venous blood appears to be a dilution of tracer by lactate (or alanine) released from the perfused tissues into the venous system. This decreases the specific activity of the metabolite and leads to increased estimates of turnover. Reilly and Chandrasena (1977) found a mean difference in lactate concentration between the arterial and venous system to be 30%. Thus, it appears to be quite necessary to measure the A-V difference across the tissues in consideration before applying a continuous infusion technique for measurement of metabolite turnover. In the liver, the situation is much complicated by the fact that the portal vein supplies as much as 70% of its perfusion and the arterial system only about 30%. Thus, the most correct measure of specific activity presented to the liver would be a mixture of portal and arterial blood specific activity (Chiasson et al, 1977). In the case of alanine, this difference is important since the gut releases alanine to the portal vein; arterial blood would present quite a different specific activity.

Ruderman et al (1976) have stated that the major gluconeogenic precursors in man are amino acids, lactate and glycerol. Of the amino acids, alanine has received major attention as it accounts for approximately 50% of amino acid release from muscle and an equivalent percent of amino acids extracted by the splanchnic bed. Felig (1975) has calculated that the alanine contribution to the glucose pool in

postabsorptive man is approximately 50% of that observed for lactate.

The effects of fasting and diet on lactate and alanine metabolismhave been studied in rats, dogs and man. In 24 hr fasted man, Kreisberg et al (1970) found a 30% reduction in glucose pool size, turnover, and oxidation; lactate turnover was unchanged, despite decreased conversion of glucose to lactate. The conversion of lactate to glucose increased 33% and represented 36% of lactate turnover. The fraction of glucose from lactate increased 2-fold. They postulated that the combination of decreased glucose conversion to lactate and increased lactate to glucose with unchanged blood lactate concentration and turnover means that lactate was increasingly synthesized from substrates other than glucose.

Freminet et al (1975a,b; 1976a,b) studied the effects of feeding and fasting on various parameters of lactate and glucose interrelationships in rats. Lactate turnover decreased progressively with fasting from 24-72 hr, then returned to fed control values with refeeding as did the % lactate turnover oxidized. When they used the Depocas-DeFreitas model in fed rats, they found that 17% of glucose turnover comes from lactate, while 28% of lactate turnover goes to glucose. They also found that the Cori cycle represented only 20-30% of glucose recycling, measured as the difference between ^{3}H and ^{14}C glucose turnover in rats. They also studied the effect of fasting on the Cori cycle. Fractionally, lactate to glucose almost doubled, glucose to lactate remained the

same, and lactate and glucose oxidized decreased. The Cori cycle increased from 10-20% in fed to 25-30% in fasted animals.

Several studies have been performed with dogs. Belo et al (1977) studied the effects of different diets on lactate and alanine turnover. While alanine turnover and pool size increased with the high protein diet, lactate pool was not influenced by diet or fasting. Dogs fed the high carbohydrate diet had low lactate to glucose in the fed state, which increased after fasting. The high protein diet decreased lactate to glucose but increased alanine to glucose. Forbath and Hetenyi (1969) showed that 14-20% of lactate comes from glucose in normal fasted dogs, while 11-18% comes from glucose in diabetic dogs.

Alanine has been shown to be an important glucose precursor in vivo in the dog (Belo et al,1977), man (Felig and Wahren,1974;Felig,1975; Cahill,1976), and lamb (Prior and Christensen,1977). In vivo turnover studies have not been done in the chicken; however, Veiga et al (1975,1977) have studied U-^{14}C-alanine incorporation into glucose after a single injection to both chickens and black vultures. The vulture, a carnivorous bird, showed higher rates of incorporation (umoles/g liver) than the chicken. Fasted vultures had decreased incorporation compared to fed vultures, while fasted chickens had higher rates than fed chickens. This type of response is somewhat similar to that of dogs found by Belo et al (1977), where plasma

alanine was higher in animals on the high protein diet, as was alanine turnover.

Bergman and Heitman (1978) recognized that there are two approaches for studying gluconeogenesis from amino acids: 1) using ^{14}C tracers as a minimum estimate for whole body conversion since some ^{14}C can cross over to the TCA cycle from oxaloacetate 2) arterial-venous differences, a maximum estimate, since the liver uses amino acids for protein synthesis also. The present work utilizes the first approach.

The Use of Isolated Hepatocytes

The use of isolated liver cells for the study of metabolic processes is advantageous for several reasons: 1) the response of the parenchymal cells can be separated from that of structural cells 2) the substrate and hormone additions can be made independently to test the effect of single additions, or added in physiological proportions.

Howard et al (1967), Howard and Pesch (1968), and Berry and Friend (1969) pioneered work with isolated rat hepatocytes, and since that time many modifications in technique have been made. Wagle and Ingebretsen (1975) reported detailed isolation and metabolic studies of rat hepatocytes. They studied net glucose production from various substrates in cells from fed and fasted rats. The rates of glucose production from highest to lowest were: fructose, lactate, pyruvate, glycerol, galactose, alanine, and succinate. Compared to the perfused liver, they assessed

the isolated cells to produce twice as much glucose from lactate, and slightly more from alanine, pyruvate, and galactose. These authors and Hems et al (1966) suggested that a stringent test of metabolic integrity of cells is the production of glucose from lactate, since this process involves integration of control between the mitochondria and cytosol and also the interchange of metabolites between them. Wagle and Ingebretsen (1975) also calculated that their isolated hepatocytes produced glucose at 50-90% the in vivo rate with 10 mM lactate or fructose.

Jeejeebhoy and Phillips (1976) have reviewed the entire technique of hepatocyte isolation and suggested the criteria for assessing normal hepatocyte function after isolation. They cited morphological integrity, functional membrane barrier, and normal protein synthesis and glucose production as essential for "normal" liver cells. The specific criteria included: spherical shape of cells, representation of all liver lobes, exclusion of trypan blue dye, minimal leakage of cell enzymes, and normal RNA and ATP levels. They also compared various buffers, ion concentrations, oxygenation, and incubation methods for short and long term cultures.

Isolated cells have been used successfullly to study gluconeogenesis by various investigators. Arinze and Rowley (1975) studied gluconeogenesis from guinea pig hepatocytes and the effect of redox state on glucose production in this species. The values obtained were similar to those

obtained for rats. Allan and Sneyd (1975) have used isolated rat hepatocytes to study the effects of glucagon on various gluconeogenic steps, while Veneziale et al (1976 a,b) have used this system to study the effects of hormones on gluconeogenic and glycolytic enzymes. Story et al (1976) have used isolated rat cells to determine glucose and ketone production from substrates with added oleate or octanoate in the fed and fasted state. Clark et al (1976) have studied basic glucose production rates in lamb hepatocytes and the effects of glucagon and butyrate on that process, while Siess et al (1977) have used hepatocytes to determine the effects of glucagon on compartmentation and gluconeogenesis from lactate in the rat. Zaleski and Bryla (1977) have used cells to determine the effects of oleate, palmitate, and octanoate in rabbits.

The use of the chicken isolated hepatocyte has not been as extensive as that of the rat, but Capuzzi et al (1971) successfully isolated adult chicken hepatocytes by initial perfusion and subsequent incubation with collagenase and found them metabolically comparable to rat cells. Goodridge (1973) isolated embryonic chick hepatocytes by a simpler procedure and found them to be a useful model in the study of avian fatty acid synthesis. However, Dickson and Langslow (1975), Anderson and Langslow (1975), and Dickson and Langslow (1977) first reported studies of glucose production in isolated chicken hepatocytes. They found that damaged cells accounted for 25% of cells in fed and 34%

in fasted chickens. In 24 hr fasted chickens, endogenous glucose production was low, as was ATP concentration. However, the cells' ability to synthesize glucose and sensitivity to hormones suggested that they were physiologically competent. The percent of competent cells decreased with time based on trypan blue dye exclusion, and correlated with increases of cellular enzymes in the media. They also found that lactate was the best glucose precursor in chick hepatocytes, followed by pyruvate, alanine, and glycerol. Cells from starved birds responded to glucagon by increasing the conversion of lactate to glucose. Dickson and Langslow (1977) further studied glucose production in the chick. Several additional substrates were characterized--aspartate, dihydroxyacetone, glyceraldehyde, malate, and succinate. They also supplied cytosolic reducing equivalents in the case of pyruvate by adding sorbitol, xylitol, ethanol, or glycerol. They noted increased glucose production over that from pyruvate alone. The work presented in this thesis examines some additional substrates and the effect of supplying mitochondrial reducing equivalents, as well as the above mentioned parameters.

The Role of Redox State and Phosphoenolpyruvate Carboxykinase Distribution

Gumaa et al (1971) have provided an excellent review of the theory and methods for redox state calculations through metabolite measurement. The basis for the evaluation of compartmental metabolite concentrations is the knowledge

that total cell concentrations of metabolites may be misleading. The concentration of effectors of enzymes should be known in close proximity to the enzyme, or at the very least, in the same compartment. The most applied method for metabolite measurement is the "freeze clamp" procedure (Hess,1974), which fixes cell constituents in a state close to the "in vivo" state, but does not allow the separation of organelle constituents. Metabolite concentrations in freeze clamped whole cells may be used to derive compartmental redox state estimates via the calculation of the nicotinamide nucleotide redox states from related dehydrogenases and their reactants (Gumaa et al,1971).

Nicotinamide adenine dinucleotide (NAD) is mostly oxidized in the cell, while nicotinamide adenine dinucleotide phosphate (NADP) is largely reduced (Gumaa et al,1971). Since the inner mitochondrial membrane is impermeable to nicotinamide nucleotides, a spatial compartmentation of these metabolites with distinct redox states was proposed (Gumaa et al,1971).

These same investigators have also presented the common criteria for calculation of nicotinamide nucleotide redox states through the "redox metabolite indicator method"; 1) the NAD pool and the metabolite pair should be related by a dehydrogenase located in a particular cellular compartment 2) the dehydrogenase activity should be high in relation to hydrogen flux such that reactants are at or near equilibrium 3) the reactants should be either uniformly distributed in the cell or confined to the compartment of the enzyme.

In establishing the cytosolic $NAD^+/NADH$ ratio, several dehydrogenases and their metabolite pairs have been proposed to meet these criteria--lactate dehydrogenase (EC 1.1.1.27), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), and malate dehydrogenase (EC 1.1.1.37). The redox ratios calculated from these agree in certain metabolic states, but discrepancies have been found (Willms et al,1970). Since glycerol-3-phosphate dehydrogenase is not always sufficiently active and malate dehydrogenase is both cytosolic and mitochondrial, the lactate/pyruvate ratio mediated by lactate dehydrogenase is usually chosen for the estimation of cytosolic redox state. The $NAD^+/NADH$ ratio can be calculated from the lactate/pyruvate ratio by the equation:

$$NAD^+/NADH = Pyr/Lact \times 1/K_{LDH}$$

assuming that lactate and pyruvate are evenly distributed in the cell.

The mitochondrial redox ratio may be calculated by the β-hydroxybutyrate/acetoacetate ratio or the glutamate/α-ketoglutarate, NH_3 ratio. Most commonly the β-hydroxybutyrate/acetoacetate ratio is used, although this calculation may be incorrect in certain circumstances (Sylvia and Miller,1973).

Redox state and compartmentation of metabolites are especially important in studies of gluconeogenesis, as the process is both mitochondrial and cytosolic. Gluconeogenesis from many three carbon intermediates begins with conversion to pyruvate, mitochondrial entry, and carboxylation to

oxaloacetate via pyruvate carboxylase (EC 6.4.1.1). In all species studied, this enzyme is mitochondrial. Alternatively, some substrates may be converted directly to oxaloacetate in the mitochondria. The disposition of oxaloacetate depends, in a species specific way, on the distribution of phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) and metabolite flux in the cell. Oxaloacetate itself is highly impermeable to the inner mitochondrial membrane.

The roles of PEPCK distribution, oxaloacetate disposition, transmitochondrial flux, and redox state are of paramount importance in discussing various species responses in gluconeogenesis. PEPCK is an enzyme which converts oxaloacetate (OAA) to phosphoenolpyruvate (PEP), a high energy intermediate in glucose production. It is a key regulatory enzyme in the gluconeogenic pathway (Garber and Hanson, 1971 a,b; Hanson,1974). PEPCK is almost completely cytosolic in the rat, mouse, and hamster; almost entirely mitochondrial in the chicken, pigeon, and rabbit; and equally divided in the guinea pig, cat, and human (Hanson,1974). As the permeability of the inner mitochondrial membrane is metabolite specific (Meijer and VanDam,1975), this distribution has important metabolic consequences.

Metabolite concentrations affecting enzymes may thus themselves be regulated by specific permeability and compartmental redox states. Both cytosolic and mitochondrial redox state and PEPCK distribution are important in regulating gluconeogenesis and in creating species differences

in that regulation (Garber and Hanson,1971a,b; Arinze et al,1973; Willms et al,1970).

Once mitochondrial OAA is formed, its fate varies by PEPCK distribution and the redox state of the mitochondria. In the rat, PEPCK is mainly cytosolic. Intramitochondrial OAA is unable to traverse the inner membrane as such, thus is removed from a key enzyme in its metabolism. The problem is solved by intramitochondrial conversion of OAA to aspartate or malate. Both transfer carbon into the cytosol in the rat (Parilla and Parilla,1976). In addition, malate also carries reducing equivalents necessary for the reduction of 1,3 diphosphoglycerate in the reversal of glycolysis. Which metabolite is the transfer form of OAA depends on the gluconeogenic substrate. When lactate is the substrate, its conversion to pyruvate produces NADH sufficient for glycolytic reversal (Hanson,1974). Hence, aspartate transfers only carbons to the cytosol. In the case of pyruvate, no NADH is produced in cytosolic metabolism, so malate transfers both carbon and reducing equivalents into the cytosol.

How does the redox state of the mitochondria affect the process? The addition of fatty acids or β-hydroxybutyrate to liver preparations has been used to promote intramitochondrial NADH production and thus alter redox state (Williamson et al,1969; Arinze et al,1973). Soling et al (1970) perfused livers of 48 hr fasted rats with 20 mM lactate and 2 mM oleate and found significantly increased glucose production in the presence of oleate. The rat livers produced

significant amounts of ketones concurrently. With lactate as a glucose precursor, sufficient cytosolic NADH should be available. Therefore, it is likely that fatty acid stimulation in rat liver occurs between pyruvate and PEP. A more reduced redox state in the rat liver is therefore favorable to increased glucose production. Octanoate (0.2 mM) also increases glucose production from lactate, pyruvate, and alanine in rat liver (Arinze et al,1973). Again, mitochondrial redox state was more reduced.

The mechanism of increased glucose production in rats under these conditions has been ascribed to acetyl CoA dependent activation of pyruvate carboxylase, increased mitochondrial reducing equivalents, or both (Arinze et al,1973), but redox shifts appear to be the best explanation. Garber and Hanson (1971a,b) showed that OAA disposition is controlled by mitochondrial redox state. When it is reduced, less PEP and more aspartate and malate are formed. As these metabolites are important in the rat in the transfer across the mitochondria, glucose production increases. Fatty acid oxidation in this species increases total OAA and malate (Soling,1974). Medium chain triglycerides increase the lactate/pyruvate ratio and the β-hydroxybutyrate/acetoacetate ratio in vivo in the rat also (Bach et al,1976). Arinze et al (1973) also perfused β-hydroxybutyrate to eliminate the effect of acetyl CoA resulting from fatty acid oxidation. β-hydroxybutyrate is metabolized to acetoacetate, producing NADH. Rat liver showed the same response as with fatty acids,

once again suggesting the importance of mitochondrial redox state.

In the guinea pig, cat, and human, PEPCK is about equally divided between the mitochondria and cytosol, and alterations in redox state influence glucose production differently than in the rat. In these species, based on data for the guinea pig, PEP may be formed in the cytosol, as in the rat, or in the mitochondria. In the mitochondria, a more reduced redox state would favor increased aspartate and malate formation and decreased PEP production (Garber and Hanson,1971a,b). Oleate (2mM) stimulated glucose production from lactate in rat liver, but inhibited it in guinea pig liver. Differences in fatty acid oxidation rate did not account for the differences in glucose production. Another study showed that after in vivo fat feeding to guinea pigs, the mitochondrial $NAD^+/NADH$ ratio was more reduced (Willms et al,1970). In the perfused guinea pig liver, prolonged exposure to fatty acids also increased the β-hydroxybutyrate/acetoacetate ratio. These data suggested that a more reduced intramitochondrial $NAD^+/NADH$ ratio might control gluconeogenesis from some substrates. Availability of OAA in the mitochondria is important in species with intramitochondrial PEPCK. Measurements of guinea pig liver showed increased oxidation with fasting (increased fatty oxidation). This shift increased OAA 8 fold in the mitochondria. It also increased PEP formation from malate, while it decreased malate formation 3 fold (Garber and

Hanson,1971a,b). PEP was the only metabolite to increase when the redox state became more oxidized. In rabbit liver, a greater proportion of PEPCK is intramitochondrial (Garber and Hanson,1971a,b). Fasted rabbits also had a more oxidized redox state than fed animals. When isolated mitochondria were subjected to varying ß-hydroxybutyrate/acetoacetate ratios, PEP synthesis from α-ketoglutarate increased with oxidized ratios, while malate formation decreased. When α-ketoglutarate and aspartate were substrates, oxidized ratios also increased PEP synthesis and decreased that of malate and citrate.

Soling et al (1970) compared the distribution of PEPCK and the effects on gluconeogenesis in the rat, guinea pig, and pigeon. The pigeon differs from the others as it has almost totally intramitochondrial PEPCK (Soling and Kleineke, 1976). Gluconeogenesis was not increased in perfused pigeon liver with lactate and 2mM oleate over that of lactate alone. Glucose production from pyruvate was low, and was not increased with the addition of hexanoate, oleate, or xylitol. However, ethanol addition increased glucose production slightly.

Blocking aspartate aminotransferase (EC 2.6.1.1) with amino-oxyacetic acid will inhibit the conversion of aspartate to OAA. In the rat and guinea pig liver, the addition of this inhibitor decreased lactate conversion to glucose, while it had no effect in pigeon liver (Soling et al,1971). These data suggested that gluconeogenesis from lactate in pigeon

liver does not depend on aspartate transfer, consistant with the location of PEPCK in this species. Gevers (1967) suggested PEP as the mode of carbon transport out of the mitochondria in the chicken. Several other studies have corroborated that the pigeon and chicken have similar PEPCK distribution (Felicioli et al,1967; Peng et al,1973; Jo et al,1974).

Thus, in studying chicken liver, one would expect to find gluconeogenic responses similar to pigeon, rabbit, and perhaps guinea pig, based on PEPCK distribution.

GLUCOSE TURNOVER IN THE YOUNG CHICK USING VARIOUSLY LABELLED (^{3}H, U-^{14}C) GLUCOSE TRACERS

Introduction

The regulation of glucose production in the chick appears to vary from that of most mammals in several important aspects. The compartmentation of phosphoenolpyruvate carboxykinase is reported to be almost entirely mitochondrial, in contrast to most mammalian species where it is evenly distributed or almost entirely cytosolic (Hanson,1974). Gluconeogenesis in avian liver is not stimulated by long or medium chain fatty acids, whereas in the rat, glucose production is stimulated by these metabolites (Soling and Kleineke,1976). The lactate/pyruvate ratio of avian liver is reported to become more oxidized in situations of increased gluconeogenesis, in contrast to the rat, where the lactate/pyruvate ratio becomes more reduced (Leveille et al,1975). This ratio is often taken to be a measure of the cytosolic NADH/NAD ratio (Gumaa et al,1971). Glucose turnover, percent glucose carbon recycling, and body glucose mass have also been found to be higher in the mature chicken than in most mammals (Belo et al,1976b).

The purpose of the present study was to begin to characterize the rate and control of glucose production in the young chick. Although several studies have been conducted comparing various double labelled glucose tracers for estimation of glucose turnover in other species, baseline data for the young chick have not been established. Estimates of "futile cycling" at the glucose - glucose-6-phosphate and fructose-6-phosphate - fructose-1,6-diphosphate stages can also be obtained with certain of the double labelled tracers. It was also of interest to compare the results obtained for

various labelled tracers in estimation of glucose metabolism when a relatively large number of animals under similar circumstances were used.

Materials and Methods

Tracers. Glucose tracers were purchased from New England Nuclear (Boston, Mass.) or Amersham-Searle (Arlington Heights, Ill.). The U-^{14}C was diluted in one batch and used for all experiments. Each tritiated glucose was separated in sufficient quantity for all experiments, then mixed with the U-^{14}C preparation. Aliquots of each tritiated tracer mixed with U-^{14}C were divided and frozen until used. The purity of glucose was determined by converting glucose to glucose-6-phosphate, retaining it on a Dowex acetate column, and eluting it with 4 N formic acid (Katz et al,1975).

Animals. Young male chicks (Fairview Farms, Remington,Ind) weighing 700-900 g were housed in pens with raised wire floors and fed a commercial high-carbohydrate diet (Master Mix Starter and Grower, Central Soya, Ft. Wayne, Ind). Water was available at all times. Chicks were fasted 24 hr prior to the tracer comparisons, direct body glucose determinations, and $^{3}H_2O$ incorporation into body glucose.

Experiment 1. Indwelling catheters (Becton-Dickinson, Rutherford, N.J.) were fixed in the jugular vein of the fasted chicks and secured with cloth tape. Approximately 1 hr later, chicks were injected through the catheter with 5 μCi of U-^{14}C glucose simultaneously with 40 μCi of either 2-tritiated (2T), 3T, 4T, 5T, or 6T glucose in 2 ml of

saline. Blood samples (1 ml) were obtained through the catheter at appropriate time intervals up to 5 hr post injection. Initial samples were taken at 2 minute intervals. The volume of blood withdrawn was replaced by saline and a total of approximately 15-20 ml was drawn from each chicken. Blood was collected in chilled tubes containing heparin and sodium fluoride. Plasma was separated and deproteinized with $Ba(OH)_2$ and $ZnSO_4$.

Glucose was separated from its metabolites by passing the deproteinized plasma through a 1 x 5 cm column containing equal mixtures of Dowex 1X8, Cl^- and Dowex 50X8, H^+. The column eluate was lyophilized, dissolved in .5 ml of H_2O, and scintillation cocktail (3a70, Research Products International, Elk Grove Village, Ill.) added. Liquid scintillation counting was done on a Packard Tri Carb scintillation spectrophotometer. Spillover of ^{14}C into the ^{3}H channel was calculated. Efficiency of counting was obtained with an external radium standard.

Plasma glucose concentration was also determined (Glucostat, Worthington Biochemical, Freehold,NJ), and specific activity of ^{14}C and ^{3}H glucose in the plasma was calculated.

Experiment 2. Either 40 μCi or 400 μCi of tritiated water in 5 ml saline was injected into a wing vein of 10 fasted chickens to determine the amount of cycling from the body water pool to glucose. Five hours after injection, a 10 ml blood sample was obtained via heart puncture. The plasma was extracted and deproteinized with 75-80% (v/v) ethanol and

boiled for a few minutes. The sample was centrifuged and the lipid removed with 5 volumes of chloroform. The upper layer containing the glucose was passed over the columns as in experiment 1. The samples were dried and redissolved in water three times to assure complete evaporation of tritiated water. The samples were then counted by the liquid scintillation procedure for tritium, using the channels ratio method to determine efficiency.

Experiment 3. The total glucose mass of 10 chicks was estimated. Chickens were fasted as in experiment 1, then killed by cervical dislocation and immediately submerged in liquid nitrogen (10 seconds elapsed between dislocation and immersion). The frozen carcasses were shattered and passed through a meat grinder until homogenous. Samples of 100 g were used for glucose extraction. Three volumes of hot water were added and the mixture heated to 90-95°C, then centrifuged at 27000 x g for 30 minutes. The extraction of the pellet was repeated three times, the total volume of all extracts combined, and glucose determined as in experiment 1, with dilution modifications.

Calculations. The results were calculated by the graphical method (Katz et al, 1974a,b). The rate of glucose utilization, percent recycling, transit time and glucose mass were calculated.

Statistical Analysis. The results were analyzed by analysis of variance for a completely randomized design. Tukey's omega procedure was performed where significant treatment

differences were found (Steel and Torrie,1960).

Results

Plasma glucose values (not shown) were constant during sampling for any one chick, but ranged from 185-300 mg/100 ml among chicks fasted one day, which is much higher than values reported for most mammalian species (Bell,1971a).

Table 1 contains the values for all parameters of glucose metabolism calculated. The glucose replacement rate (rate of utilization) based on 2T-glucose was significantly higher than that from either 5T- or 6T-glucose. A 25% difference existed between the 2T-glucose replacement rate and the 6T-glucose replacement rate, suggesting the presence of a "futile cycle" between glucose and glucose-6-phosphate. The 5T-glucose replacement rate was not significantly higher than the 6T-glucose replacement rate, suggesting no apparent "futile cycle" in the chicken between fructose-6-phosphate and fructose-1,6-diphosphate. No significant differences were found between 3T- and 6T-glucose, or between 3T- and 4T-glucose replacement rates. No significant difference was found among U-^{14}C-glucose replacement rates. Figure 1 presents the ^{3}H/^{14}C ratios of the tracers for the first 2 hr of the experiments and indicates the similarity of 3T-, 4T-, 5T-, and 6T-glucose tracers in these experiments.

The percent of glucose carbon recycling (Table 2) was significantly higher for 2T-glucose than for the other tracers. No significant differences were found in estimates of tricarbon recycling obtained with 3T-, 4T-, 5T-, or 6T-glucose.

Table 1. Glucose replacement rates in chicks, mg/min/kg*.

Glucose tracer	^{3}H (T)	U-^{14}C	Significant differences†
2T, U-^{14}C (9)	21.4 ± 1.0	9.1 ± 0.4	2T vs 5T, 6T ($P<0.05$)
3T, U-^{14}C (5)	15.8 ± 0.5	8.4 ± 0.2	
4T, U-^{14}C (10)	15.6 ± 1.0	9.2 ± 0.9	
5T, U-^{14}C (5)	17.0 ± 1.6	10.4 ± 1.3	
6T, U-^{14}C (10)	16.0 ± 0.7	5.6 ± 0.6	

*Values are mean ± SEM for 5-10 chicks (700-900g) per group as indicated ( ).

†No significant differences were found for U-^{14}C values. Comparisons made for ^{3}H values were: 2T vs 5T, 2T vs 6T, 3T vs 6T and 3T vs 4T.

Table 2. Glucose recycling in chicks*.

Glucose tracer	%	Significant differences†
2T, U-^{14}C (9)	57 ± 2	2T vs 5T, 6T ($P<0.05$)
3T, U-^{14}C (5)	47 ± 2	
4T, U-^{14}C (10)	40 ± 3	
5T, U-^{14}C (5)	40 ± 2	
6T, U-^{14}C (10)	40 ± 2	

*Values are mean ± SEM for 5-10 chicks (700-900g) per group as indicated ( ).

†Comparisons were made for ^{3}H (T) values: 2T vs 5T, 2T vs 6T, 3T vs 6T and 3T vs 4T.

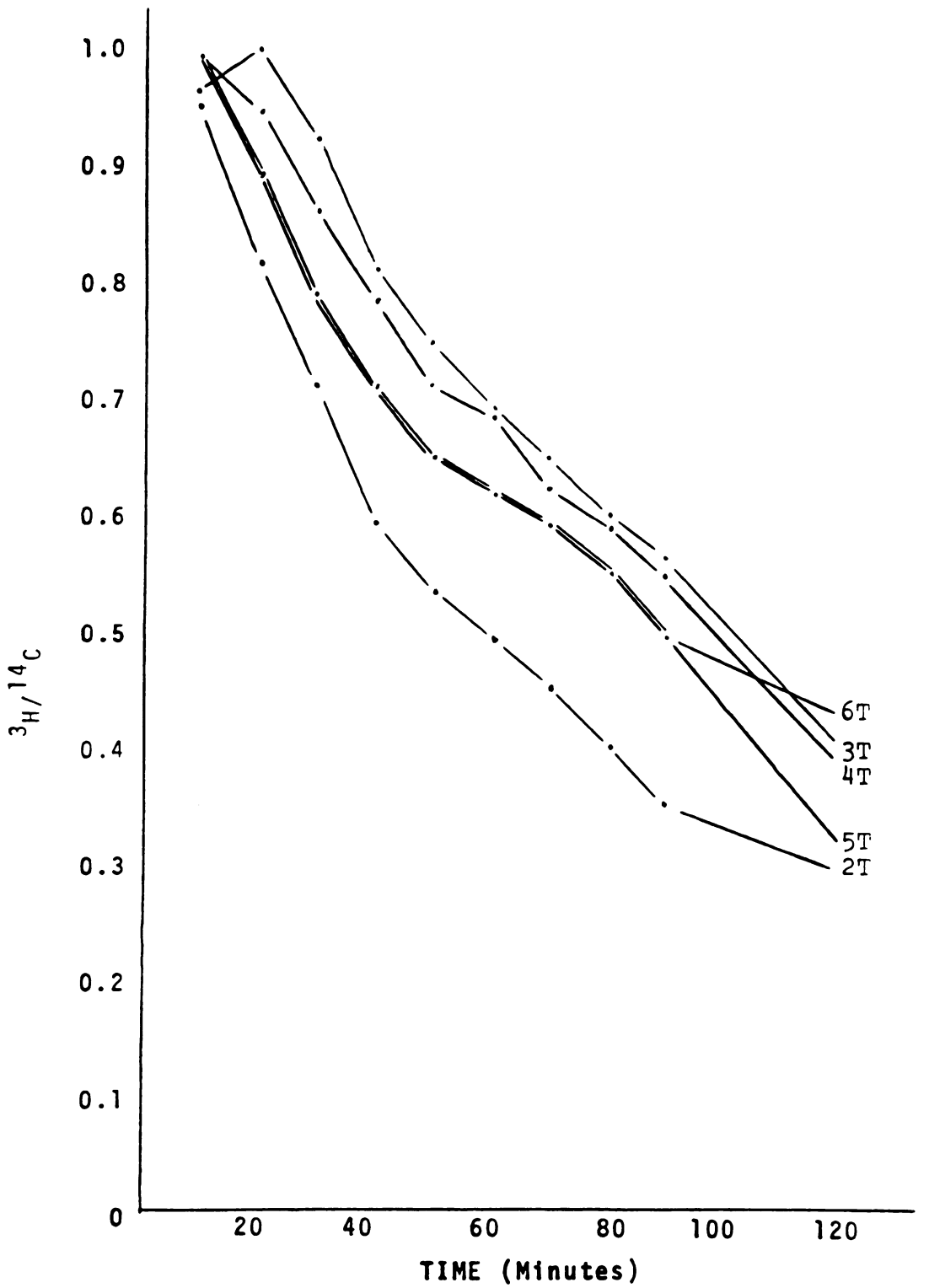

Figure 1. $^{3}H/^{14}C$ ratios in plasma glucose of chicks.

The mean transit times, or average time a glucose molecule remains in the body pool as glucose, are presented in Table 3. A significant difference was found between 2T- and 5T- glucose transit times, and between 2T- and 6T-glucose transit times, but no differences were found between 3T- and 4T-glucose transit times, nor between 5T- and 6T-glucose transit times. A significant difference occurred between 3T- and 6T-glucose transit times, but not among transit times based on U-^{14}C-glucose.

Glucose mass values are also presented in Table 4. No significant treatment differences were found. The calculated values agree with the body mass determined from direct carcass analysis. When mass was roughly estimated based on avian extracellular water volume (Freeman,1971) and plasma glucose concentration, a value of 800-1200 mg/kg was obtained.

When tritium is lost from glucose, it becomes part of the body water pool (Katz et al,1974a,b). The extent of re-incorporation of this tritium into glucose was examined and the results are presented in Table 5. The extent of incorporation (dpm/mg glucose) was low at both levels of $^{3}H_2O$ injection in comparison to the dpm/mg glucose during the first 3-4 hr of sampling. Typical glucose specific activities in plasma at different times after injection of labelled glucose were: 2 min--100,000; 15 min--24,000; 45 min--11,000; 120 min--2200; 210 min--800; 300 min--600 dpm/mg glucose.

Discussion

The choice of tritiated glucose tracer for use in

Table 3. Mean transit times of variously labelled glucoses in chicks, minutes*.

Glucose tracer	^{3}H (T)†	U-^{14}C†	Significant differences
2T, U-^{14}C (9)	50 ± 3	103 ± 4	2T vs 6T ($P<0.05$)
3T, U-^{14}C (5)	46 ± 1	111 ± 6	3T vs 6T ($P<0.05$)
4T, U-^{14}C (10)	51 ± 2	107 ± 2	
5T, U-^{14}C (5)	58 ± 6	112 ± 3	
6T, U-^{14}C (10)	61 ± 3	107 ± 4	

* Values are mean ± SEM for 5-10 chicks (700-900g) as indicated ( ).

† No significant differences were found for U-^{14}C values. Comparisons made for ^{3}H values were: 2T vs 5T, 2T vs 6T, 3T vs 6T and 3T vs 4T.

Table 4. Glucose mass in chicks, mg/kg*.

Glucose tracer	^{3}H (T)†	U-^{14}C†
2T, U-^{14}C (9)	1086 ± 107	990 ± 65
3T, U-^{14}C (5)	734 ± 31	934 ± 68
4T, U-^{14}C (10)	798 ± 53	988 ± 44
5T, U-^{14}C (5)	983 ± 134	1170 ± 167
6T, U-^{14}C (10)	968 ± 53	995 ± 34

* Values are mean ± SEM for 5-10 chicks (700-900g) as indicated ( ).

† No significant differences were found among U-^{14}C values; nor among ^{3}H (T) for the comparisons made: 2T vs 5T, 2T vs 6T, 3T vs 6T and 3T vs 4T.

estimation of glucose turnover is complicated by the different fates of the ^{3}H atoms attached to the various carbons of glucose. It appears to be generally agreed now, that 2T-glucose may overestimate the rate of glucose production(replacement rate) due to the presence of an hepatic futile cycle at the glucose--glucose-6-phosphate steps (Dunn et al,1976; Anwer et al,1976). This futile cycling leads to a rapid loss of tritium from position 2 of glucose and thus, overestimates the replacement rate. It is less clear whether a futile cycle exists at the fructose-6-phosphate--fructose-1,6-diphosphate stage, which would lead to an overestimation of replacement rate by 5T-glucose (Hue et al,1974; Dunn et al, 1976; Anwer et al,1976; Clark et al,1975; Clark et al,1973). Our results do not indicate an overestimation of replacement rate by 5T-glucose compared to 6 T-glucose (17.0 vs 16.0 mg/min/kg). Thus, there appears to be little futile cycling at the fructose level in young chicks. A problem with the use of 5T-glucose has recently been cited (Rognstad et al, 1975). A significant retention of tritium was found when 5T-fructose was metabolized which the authors concluded could influence interpretation of studies using 5T-glucose tracers. Our assumption in these experiments was that isotope discrimination was equal for all groups.

Most workers are now in agreement that 6T-glucose represents the best choice of ^{3}H glucose tracers for determination of glucose turnover (Dunn et al,1976; Anwer et al,1976; Katz et al,1976; Belo et al,1976a) as it represents Cori

Table 5. ^{3}H incorporation into plasma glucose from $^{3}H_2O$*.

Dose	dpm/mg glucose
40 µCi	60 ± 7
400 µCi	70 ± 6

* Values are mean ± SEM for 5 chicks (700-900g) taken at 5 hours post injection.

cycle activity and appears not to be involved in futile cycling.

Our results show little difference between 3T-,4T-, 5T- and 6T-glucose replacement rates and recycling in vivo. This suggests that the true rate of glucose production by the liver and kidneys may be measured by all four tracers. The close agreement between 3T- and 6T- values has been observed by others (Hue et al,1974; Dunn et al,1976; Judson and Leng,1972) and Clark et al (1975) found 3T- and 4T- values similar in perfused rat liver. Katz et al (1975) found little difference among 3T-, 5T-, and 6T-glucose replacement rates and percent recycling in rats in vivo, but 5T- values were greatest, followed by 3T-, then 6T- values. Dunn et al (1976) found 2T- and 5T-glucose replacement rates and percent recycling greater than that of 3T- and 6T- glucose replacement rates, the latter two being similar in rabbits in vivo. Judson and Leng (1972) found little difference in 2T-, 3T-, and 6T-glucose replacement rates in fed sheep. Anwer et al (1975) found 3T- replacement rates similar to 6T- values in ponies. Thus, it appears that use of 2T-glucose leads to the highest replacement rates and percent recycling, perhaps due to the presence of an hepatic futile cycle. The order of rates of 3T-, 4T-, 5T-, and 6T-glucose tracers appears to differ depending on the species and experimental circumstances. To our knowledge, no one else has compared all tracers simultaneously in vivo in one species.

The young chicken's rate of glucose turnover based on

2T-glucose was very high, both compared to mature chickens and to mammalian species. Katz et al (1976) and Dunn et al (1976) have expressed the percent of 2T-glucose replacement believed due to futile cycling at the glucose-glucose-6-phosphate step, as the difference in the detritiation of 2T-glucose and 6T-glucose. This estimate was 25% in this study, or a 5.4 mg/min/kg body weight difference in the two replacement rates. This would indicate a much higher futile cycle activity at the glucose-glucose-6-phosphate stage than reported for other species (Dunn et al,1976; Anwer et al,1976; Katz et al,1976; Belo et al,1976b). The replacement rates of 3T-, 4T-, 5T-, and 6T-glucose still reflect a rapid replacement rate. A question remaining to be answered for the chick is: which tissues of the fasted chick utilize large amounts of glucose? Bell (1971) has reviewed data indicating that little glucose is utilized by red blood cells in chickens, contrary to the situation in mammals where glucose is the sole substrate of red cells. Lehr et al (1976) have shown rapid incorporation of labelled glucose into brain amino acids of post hatched chicks.

Regardless of the tracer used, the chick appears to have a great capacity of recycling tricarbon units originally derived from glucose. Values reported for other species are: 12-16% in ponies(Anwer et al,1976), 14% in mature dogs (Belo et al,1976a), 19% in rabbits (Dunn et al,1976), 14% in rats (Katz et al,1976), and 27% in mature chickens(Belo et al, 1976b), compared to 40% in the young chicks in this study.

The transit time was generally correlated with replacement rate in our study, that is, the shorter the transit time, the faster the replacement rate. The transit times for 2T- and 3T-glucose were the most rapid, while 6T-glucose was the slowest. The transit time for 5T-glucose was slower than 2T-glucose which might reflect tritium retention (Rognstad et al,1975). The transit time for 3T-glucose was similar to 2T-glucose rather than 6T-glucose, which was not expected. No explanation for this is readily apparent. Dunn et al (1976) found 3T-glucose transit times and 2T-glucose transit times similar to 6T-glucose transit times in rabbits in vivo. Katz et al (1976) did not find widely different transit times for 2T-, 3T-, 5T-, and 6T-glucose tracers in rats in vivo. The similarity of transit times for U-^{14}C indicated the repeatability of the techniques among different treatments in our study.

Estimations of minimal glucose mass by our methods agreed closely with the direct determination of body glucose mass. The agreement was similar for estimates based on both ^{3}H and U-^{14}C glucose. These values correspond to the $Mass_{min}$ of Katz et al (1974 ab), and suggest that glucose catabolism occurs in close proximity to the sampling pool in the young chicken. The agreement of the calculated and the direct methods lends support to the validity of the graphical method of analysis. The glucose mass of the chicken(mg/kg) was much higher than in dogs--280(Belo et al,1976a), rats--400 (Katz et al,1976), rabbits--274 (Dunn et al,1976),

ponies--130-150 (Anwer et al,1976), and mature chickens--270-700 (Belo et al,1976b).

The high body glucose mass in the chick compared to mammalian species correlated well with the higher plasma glucose levels, which ranged as high as 300 mg/ml in chickens. The directly determined body mass also agreed with the calculated values. Directly determined values probably do not include any glucose derived from glycogen, since the animals had been fasted 24 hr prior to the determination of glucose, a time which, in other species, has been shown to deplete hepatic glycogen (Ruderman et al,1976). The method of Katz et al (1974 a,b) also has been found to give body glucose masses for other species similar to those obtained with other methods. Belo et al (1976a) found body glucose masses in mature chickens to be similar using the graphical method and exponential methods (Shipley and Clark, 1972). They found high body glucose masses in chickens with both methods.

Rognstad et al (1974) reported variability in the in vitro incorporation of tritium into glucose from ^3H_2O. Neither 40 μCi nor 400 μCi showed significant incorporation of tritium from ^3H_2O into glucose in our experiments. The specific activity values from 2 min to 3 hr after injection would not be affected greatly by the addition of this number of counts from water, as the activity at these times is relatively high. After this period (4-5 hr post injection), the plasma specific activity drops and reincorporated counts could add more activity on a relative basis than at earlier times.

However, these times do not contribute much of the total area under the specific activity curve, and thus would not affect the calculations significantly.

In summary, these experiments examined the parameters of glucose metabolism in young chicks utilizing various labelled tracers. Although 2T-glucose led to the highest replacement rates and percent recycling, no difference was found among the other tracers. All the tracers were found to give a valid estimate of glucose mass compared to that directly determined. Little tritium from ^3H_2O was found to be reincorporated into body glucose. The young chicken was found to have a high rate of glucose turnover and percent recycling compared to mature chickens and mammals.

THE EFFECTS OF FASTING ON BODY COMPOSITION, GLUCOSE TURNOVER, METABOLITES, AND ENZYMES IN THE CHICKEN

The fasting state in the human and the dog is characterized by decreased plasma glucose levels (Owen et al,1969; Cahill et al,1966; Brady et al,1977), decreased glucose turnover (Brady et al,1977); Kreisberg et al,1970), decreased plasma gluconeogenic precursors (Brady et al,1977; Felig et al,1969), and increased blood ketone levels (Cahill et al,19669; Brady et al,1977; Wiener et al,1971). The subcellular location of a key regulatory enzyme in hepatic glucose production, phosphoenolpyruvate carboxykinase (PEPCK), is also similar in these two species, divided approximately equally between the cytosolic and mitochondrial compartments (Soling and Kleineke,1976; Belo et al,1976). The subcellular location of PEPCK influences the flux of carbon and reducing equivalents between mitochondria and cytosol in the gluconeogenic state in many species: rat, rabbit, guinea pig, and pigeon (Hanson,1974; Soling and Kleineke,1976; Garber and Hanson,1971a,b; Arinze et al,1973; Soling et al,1970; Soling et al,1973).

The chicken, however, has been reported to maintain plasma glucose levels and glucose turnover rates during fasting (Belo et al,1976b; Evans and Scholz,1971). An oxidized mitochondrial redox state is associated with increased phosphoenolpyruvate (PEP) and glucose production in guinea pig liver, in which 50% of PEPCK is mitochondrial, and it has been proposed that in other species with mitochondrial PEPCK, an oxidized mitochondrial redox state would be favorable to PEP production (Soling and Kleineke,1976). This is in contrast

to the rat, where a reduced mitochondrial redox state has been shown most favorable to PEP production (Soling and Kleineke,1976). The pigeon and the chicken also possess almost entirely intramitochondrial PEPCK in liver, which in the pigeon, has been reported to remain constant with fasting (Soling et al,1973). Thus, it was of interest to examine the effect of fasting on body composition, glucose turnover, metabolites used in redox estimation, gluconeogenic metabolites, and enzymes involved in glucose production and mediating redox reactions in the chicken.

Materials and Methods

Animals. One day old male broiler chicks were obtained from a commercial source and fed a stock carbohydrate diet (See Chapter 1). Water was available ad libitum; room lights were on 24 hr per day. When the chickens reached 1200 g, the experiments were started. Measurements were made in the fed state, and at 1, 4, and 8 days of fasting.

Experiment 1. Chickens (7 per group) were killed on the appropriate days and whole body composition was obtained. The carcasses were dried 48 hr at 80°, and ground twice through a meat grinder (feathers included) to a homogenous consistency. Total nitrogen (Horwitz,1960) was determined; protein was assumed to equal nitrogen x 6.25. Total fat was determined gravimetrically after chloroform:methanol extraction (2:1).

Experiment 2. Chickens (5 per group) were divided into groups upon reaching 1200 g. Glucose turnover measurements were performed on days 1, 4, and 8 of fasting. Indwelling

catheters (Becton-Dickinson, Rutherford,N.J.) were implanted 1 hr before the start of the experiment. Forty μCi of (2-^{3}H) or (6-^{3}H) glucose (specific activity-500 mCi/mmole) were injected simultaneously with 5 μCi (U-^{14}C) glucose (specific activity-180mCi/mmole). Blood samples were drawn at timed intervals and samples processed as previously described (Belo et al,1976).

Experiment 3. Chickens (10 per group) were divided into five groups. The chickens in the first group were bled from a wing vein in the fed state and at 1, 4, and 8 days of fasting. Five ml of blood were drawn from each bird in group 1 on appropriate days and placed in iced tubes containing heparin. After shaking, 2 ml of whole blood were precipitated in 8 ml of 6% trichloroacetic acid (TCA). The tubes were centrifuged at 2500 rpm for 15 minutes and the resulting supernatant neutralized with 20% KOH. The tubes were centrifuged again at 2500 rpm for 15 minutes and assays done on the resulting supernatant. Three ml of heparinized blood were also centrifuged to obtain plasma for amino acid analyses. Plasma was precipitated with 10 volumes of 20% sulfosalicylic acid. Norleucine was added as an internal standard. Analyses were performed in a lithium citrate buffer system using a Technicon amino acid analyzer.

Groups 2-5 were used for hepatic and renal metabolite determinations in the fed state and at 1, 4, and 8 days of fasting. In these groups cervical dislocation was performed simultaneously with a mid ventral incision below the sternum.

The sternum and overlying muscle were rapidly raised, the liver exposed, and freeze clamped with tongs precooled in liquid nitrogen. The whole process took no more than 5 seconds. Immediately after freeze clamping the liver, a kidney was rapidly removed, rinsed in buffer, blotted, and frozen in liquid nitrogen. Approximately 15-20 seconds elapsed before the kidney was frozen. Frozen tissues were weighed into previously tared tubes containing 6% TCA and homogenized. The homogenate was centrifuged at 10,000 x g for 15 minutes and the supernatant decanted and neutralized as the blood samples. Before analyses, supernates were treated with Florisil (Fisher Laboratory Chemicals, Fairlawn, NJ) to remove flavins (Garber and Hanson,1971 a,b).

Lactate (Gutman and Wahlefeld, 1974), pyruvate (Czok and Lamprecht, 1974), β-hydroxybutyrate (Williamson and Mellanby, 1965) and acetoacetate (Mellanby and Williamson, 1965) levels were determined by standard enzymatic techniques. In addition, glutamine and glutamate (Bergmeyer and Bernt, 1974), and α-ketoglutarate (Lund, 1974) levels were determined in kidney and liver.

Experiment 4. Chickens (5 per group) were fed or fasted 1,4 or 8 days. On the appropriate day, chickens were killed, and liver and kidney removed for enzyme analysis. Liver was homogenized in 10 volumes 0.25M sucrose containing 1mM reduced glutathione and 1mM EDTA. Kidneys were homogenized in 5 volumes of the same buffer. Homogenates were centrifuged

at 600 x g for 15 minutes, and the resulting supernatant recentrifuged at 18,000 x g for 15 minutes. The supernatant from this centrifugation was used to assay lactic dehydrogenase. The pellet from this centrifugation was resuspended in 10 volumes of ice water and sonicated (Branson Sonifier, Branson Sonic Power Co., Danbury, CT). This suspension was used for assay of phosphoenolpyruvate carboxykinase, glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase. Lactic dehydrogenase (EC 1.1.1.27)(Bergmeyer and Bernt, 1974b), glutamate dehydrogenase (EC 1.4.1.2)(Schmidt, 1974) and β-hydroxybutyrate dehydrogenase (Wilkinson, 1974) were determined by oxidation of NADH. PEPCK (EC 4.1.1.32) was determined by the method of Helmrath and Bieber (1974) as fixation of ^{14}C-bicarbonate into acid-stable malate. Enzyme activities were expressed per mg protein (Lowry et al, 1951). Data were analyzed by analysis of variance (completely randomized design). Tukey's omega procedure was used to test treatment means when significant ($P<0.05$) treatment F values were found (Steel and Torrie, 1960).

Results

Table 6 contains the results of experiment 1. Body weight decreased significantly with fasting. Liver and kidney weights also decreased significantly with fasting. Total body fat decreased between 1 and 4 days and again between 4 and 8 days of fasting. Total body protein decreased between 1 and 4 days, but remained constant from 4 to 8

Table 6. Body weight and composition of chickens fasted for 0, 1, 4, or 8 days (experiment 1)[1].

	Days fasted			
	0	1	4	8
Body weight (g)	1211±15[a] (12)	1083±14[b] (10)	922±15[c] (11)	778±18[d] (11)
Liver weight (g)	30.5±1.5[a] (10)	25.0±0.8[b] (8)	18.2±0.8[c] (11)	11.9±0.8[d] (10)
Kidney weight (g)	2.3±0.1[a] (10)	2.4±0.3[a] (8)	1.4±0.1[b] (11)	1.0±0.1[c] (10)
Total protein (g)	ND	216±5[a] (7)	197±4[b] (7)	187±4[b] (7)
Total fat (g)	ND	148±11[a] (7)	109±11[b] (6)	61±12[c] (7)

[1]Mean SEM. The number of chickens is indicated in parentheses. Means in the same line with different letter superscripts are significantly different ($P<0.05$). ND = not determined.

days. Thus, body composition data indicate that fasting chickens metabolized slightly greater amounts of protein initially and almost equal amounts of fat in each period of fasting. It was also noted that the chickens decreased voluntary activity as fasting progressed and tended to huddle together in a corner of the cage.

The results of experiment 2 are presented in Table 7. Plasma glucose remained constant during the sampling period. Tritiated glucose was used simultaneously with U-^{14}C glucose to facilitate the calculation of ^{14}C recycling (Katz et al, 1974; 1974b). The choices of tritiated glucose species used in this study were purposive. The use of 6-^{3}H glucose has been accepted as the best approximation of true hepatic glucose production (Katz et al, 1974; 1974b; Anwer et al, 1976), while the use of 2-^{3}H glucose has been proposed to allow estimation of the degree of futile cycling at the glucose - glucose-6-phosphate stage. Since both tritiated tracers were not used in each chicken, only a mean difference between the 2-^{3}H glucose replacement rate and the 6-^{3}H glucose replacement rate was obtained. This represents an approximation of futile cycling at this point in metabolism.

Glucose replacement rates were significantly greater at every time period for 2-^{3}H glucose than 6-^{3}H glucose. There were no differences at any time in the U-^{14}C glucose

Table 7. Glucose metabolism in fasted chickens[1,2] (experiment 2).

	Days fasted			SEM
	1	4	8	
Body weight (g)	1083[a]	922[b]	778[c]	16
Plasma glucose (mg/dl)	208	222	224	16
Glucose replacement rate:				
(2-^{3}H)-glucose (mg/min/kg)	13.1	11.4	13.3	1.0
(6-^{3}H)-glucose (mg/min/kg)	10.7	9.2	11.0	1.0
(U-^{14}C)-glucose (mg/min/kg)	8.3	7.6	7.8	0.5
(6-^{3}H)-glucose (mg/min/chick)	11.5[a]	8.5[b]	8.5[b]	0.7
Percent C recycling[3]	22	14	28	3
Body glucose mass:				
(2-^{3}H)-glucose (mg/kg)	588[a]	572[a]	816[b]	68
(6-^{3}H)-glucose (mg/kg)	551	538	697	68
(U-^{14}C)-glucose (mg/kg)	648	645	710	56

[1]Mean for 5 chickens. 2-^{3}H glucose replacement rate was greater than 6-^{3}H glucose replacement rate at all times. U-^{14}C values were combined for both groups as there was no significant difference between them.

[2]Numbers in the same line with different letter superscripts are significantly different ($P<0.05$).

[3]Percent recycling = 6^{3}H glucose replacement rate minus U^{14}C glucose replacement rate divided by the 6^{3}H glucose replacement rate.

replacement rates (mg/min/kg). Neither tritiated tracer suggested a decreased glucose production per kg body weight with fasting. However, glucose production per total animal decreased between days 1 and 4 of fasting as the chickens lost weight.

The percent ^{14}C-carbon recycling, which has been used to estimate the percentage of tricarbon units originally derived from glucose that return to glucose, was of the same order as found for dogs (Brady et al,1977), and slightly higher than ponies (Anwer et al,1976), rats (Katz et al,1976), and rabbits (Dunn et al,1976). It is suggested from these data that the chicken may not increase the amount of tricarbon units from glucose returning to glucose with a long fast.

The constancy of glucose metabolism is further reflected in the body glucose mass. Most values agree very closely, with the exception of the 2-^{3}H-glucose 8 day value. However, this mean was influenced by one very high value in comparison with the others.

Levels of circulating metabolites are presented in Table 8. Glycerol levels remained relatively constant with fasting. Lactate levels were higher than reported for rats and dogs (Brady et al,1977; Romsos et al,1974), with no significant change during fasting. Pyruvate levels increased significantly between fed and 1 day of fasting, but did not increase significantly thereafter. Thus, the plasma lactate/pyruvate ratio declined from fed to 1 day of fasting, but

Table 8. Circulating levels of metabolites in fed and fasted chickens[1,2] (experiment 3).

Metabolite (nmol/ml blood)	Days fasted				SEM
	0	1	4	8	
Glycerol	ND	180	130	110	40
Lactate	4750	3040	3420	4030	460
Pyruvate	185[a]	309[b]	213[b]	298[b]	20
Lactate:pyruvate	26[a]	10[b]	16[b]	14[b]	3
β-hydroxybutyrate	362[a]	2400[b]	3690[c]	2610[b]	300
Acetoacetate	187[a]	103[a]	55[b]	122[a]	20
β-hydroxybutyrate:acetoacetate	2[a]	23[b]	67[c]	21[b]	9

[1]Mean for 10 chickens. ND = not determined.

[2]Means in the same line with different letter superscripts are significantly different ($P<0.05$).

did not decline further with fasting. Blood β-hydroxybutyrate levels rose substantially from fed to 1 day of fasting; blood acetoacetate levels remained fairly constant during the fast. Consequently, the blood β-hydroxybutyrate/acetoacetate ratio increased markedly from the fed to the fasted state.

Plasma amino acid levels are presented in Table 9. Alanine and serine levels decreased slightly, but not significantly during the fast. Glycine levels remained relatively constant. The constant levels of these possible gluconeogenic precursors agrees well with constant levels of glycerol, pyruvate, and lactate and a constant rate of glucose utilization during the fast. Threonine and methionine increased between days 1 and 4, while 1/2 cystine increased between days 4 and 8. Aspartate, glutamate and glutamine, tyrosine, phenylalanine, and lysine exhibited no significant differences with fasting. As noted by others(Hill and Olsen,1963; Tasahi and Takuo,1971) plasma lysine levels were high compared to mammalian levels. Branched chain amino acids did not all respond in the same manner. Valine and leucine increased from 1 to 4 days, then changed no further. Isoleucine fell significantly with fasting. 3-methyl histidine increased markedly with fasting, probably as a result of increased muscle breakdown (Young,1970), although decreased excretion could also contribute to the rising levels. N - methyl lysine also increased with fasting. Uric acid levels tended to remain constant, in agreement with a previous study

Table 9. Amino acid and uric acid levels in the fasted chicken[1] (experiment 3).

	Days fasted			SEM
	1	4	8	
	- plasma (nmol/ml) -			
Alanine	1449	1318	1221	63
Serine	2331	2094	1873	137
Glycine	1211	1276	1394	72
½ Cystine	46[a]	44[a]	75[b]	3
Threonine	2981[a]	4258[b]	4559[b]	393
Aspartate	93	112	114	6
Glu + Gln	1034	1075	959	96
Proline	535	539	539	27
Methionine	89[a]	131[b]	144[b]	5
Tyrosine	226	216	195	10
Phenylalanine	179	203	191	7
Valine	495[a]	823[b]	594[c]	25
Isoleucine	356[a]	266[b]	263[b]	15
Leucine	406[a]	548[b]	469[a]	19
Lysine	2858	2436	2003	212
NΣ-Methyl lysine	131[a]	418[b]	661[c]	42
Arginine	390[a]	471[b]	323[a]	35
Histidine	234[a]	360[b]	175[a]	56
3-Methyl Histidine	7[a]	22[b]	29[c]	3
Uric acid	3	4	4	0.2

[1]Each value is the mean for 10 chickens. Means in the same line with different letter superscripts are significantly different ($P<0.05$).

with fasting chickens(Evans and Scholz,1971).

Since the hepatic redox state has been implicated in the control of PEP and glucose formation in some mammals (Hanson, 1974; Soling and Kleineke,1976; Garber and Hanson,1971 a,b), metabolites commonly used to estimate compartmental redox states were measured (Table 10). Liver lactate remained constant, as did liver pyruvate. The liver lactate/pyruvate ratio was not significantly different during the fast. Liver β -hydroxybutyrate increased from fed to fasted, while acetoacetate levels remained constant, thus increasing the β-hydroxybutyrate/acetoacetate ratio. Liver glutamate and α-ketoglutarate increased with fasting, as did glutamine. The glutamate/α-ketoglutarate ratio decreased with fasting.

Table 10 also contains the data for kidney metabolite levels. Lactate values tended to be higher than in liver during fasting, possibly reflecting increased anaerobic glycolysis, as this organ was not freeze-stopped as quickly as liver. Pyruvate levels were of the same order as in liver, as was the lactate/pyruvate ratio. β - hydroxybutyrate and acetoacetate levels and the β-hydroxybutyrate/acetoacetate ratio were similar to liver. α -ketoglutarate did not increase as markedly as in liver, but glutamate and glutamine were higher, possibly due to the critical role of the kidneys in maintaining acid-base balance, as well as their possible role in glucose production (Sykes,1971).

Table 10 contains the enzyme data for fed and fasted chickens. Liver and kidney PEPCK activity remained constant

Table 10. Liver and kidney metabolites in fasted chickens (Experiment 3)[1].

Metabolite (nmol/g tissue)	Days fasted 0	1	4	8	SEM
Liver					
Lactate	1570	1410	920	1340	400
Pyruvate	111	137	91	140	25
Lactate:pyruvate	14	10	10	10	2
β-hydroxybutyrate	327^{a}	1970^{b}	1370^{b}	1650^{b}	160
Acetoacetate	292	344	402	201	70
β-hydrybutyrate:acetoacetate	1.1^{a}	6^{b}	3^{ab}	8^{b}	2
Glutamate	3620^{a}	6180^{b}	4500^{ab}	6290^{b}	520
α-ketoglutarate	80^{a}	262^{ab}	402^{bc}	574^{c}	40
Glutamate:α-ketoglutarate	45^{a}	24^{b}	11^{c}	9^{c}	3
Glutamine	1090^{a}	5730^{b}	2720^{a}	2690^{a}	520
Kidney					
Lactate	1570	2130	1200	1960	280
Pyruvate	166^{a}	309^{b}	150^{a}	178^{a}	50
Lactate:pyruvate	9	6	8	11	3
β-hydroxybutyrate	261^{a}	1380^{bc}	1120^{b}	1780^{c}	140
Acetoacetate	269	216	307	191	72
β-hydroxybutyrate:acetoacetate	1^{a}	6^{b}	4^{b}	9^{b}	2
Glutamate	8600^{a}	11700^{b}	11000^{b}	15000^{c}	560
α-ketoglutarate	111^{a}	82^{a}	109^{a}	495^{b}	30
Glutamate:α-ketoglutarate	78^{a}	14^{b}	10^{b}	3^{c}	3
Glutamine	2200^{a}	6600^{b}	5100^{b}	4400^{c}	780

[1]Each value is the mean for 10 chickens. Means in the same line with different letter superscripts are significantly different ($P<0.05$)

Table 11. Enzyme activities in fasted chickens (experiment 4)[1].

Enzyme (nmol/min/mg protein)	Days fasted 0	1	4	8	SEM
Liver					
PEPCK	650	510	500	470	105
Lactate dehydrogenase	1340[a]	2240[b]	5300[c]	5440[c]	400
Glutamate dehydrogenase	92[a]	149[a]	223[b]	112[a]	22
β-hydroxybutyrate dehydrogenase	19	35	10	16	10
Protein:					
Mitochondria (mg/g tissue)	54	51	44	46	6
Cytosol (mg/g tissue)	54	49	50	55	6
Kidney					
PEPCK	772	930	875	825	101
Lactate dehydrogenase	2250	2620	2140	2360	200
Glutamate dehydrogenase	94[a]	117[a]	134[b]	78[a]	13
Protein:					
Mitochondria (mg/g tissue)	134	103	88	102	12
Cytosol (mg/g tissue)	42[a]	50[a]	74[b]	45[a]	8

[1]Each value is the mean for 10 chickens. Means in the same line with different letter superscripts are significantly different ($P<0.05$).

throughout the fast. Liver lactate dehydrogenase activity was high and increased significantly with fasting, while kidney activity remained constant. Liver and kidney glutamate dehydrogenase tended to be highest at 4 days, with no differences otherwise. Liver β-hydroxybutyrate dehydrogenase activity was low at all times. Neither liver nor kidney protein concentration varied significantly with fasting.

Discussion

The response to fasting elicited in the young chicken was the maintanence of a constant rate of glucose utilization per kilogram body weight from 1 to 8 days. In the dog there is an initial drop in glucose utilization from 1 day fast to 1 week, and a constant level from then on (Brady et al, 1977). This adaptation apparently did not occur in the chicken during the time period monitored. In the chicken, the rate of glucose utilization per kg body weight remained constant as well as plasma levels of possible glucose precursors. However, total glucose utilization per animal decreased between day 1 and 4 of fasting in chickens (present study) and dogs (Brady et al, 1977), as both groups of animals lost weight. Increased plasma levels of N-methyl-lysine and 3-methyl-histidine could be indicative of increased muscle breakdown (Young, 1970; Hardy and Perry, 1969) to provide amino acids for glucose production, energy and essential protein synthesis. Increasing levels of these

metabolites occurred concurrently with decreasing carcass nitrogen from 1 to 4 days. Plasma uric acid production did not increase significantly, an observation noted by others (Evans and Scholz, 1971).

Providing that the assumptions of tracer methodology are correct (Katz et al, 1974; 1974b) and that the difference between the replacement rate with 2-^{3}H glucose and 6-^{3}H glucose is indicative of "futile cycling"(Issekutz, 1977), it appears that the chicken possesses an active "futile cycle" or "substrate cycle" at the glucose-glucose-6-phosphate stage. Although statistics cannot be applied to the mean differences at each time period obtained in the present experiment, values obtained are quite similar: 2.3, 2.2, and 2.3 mg/min/kg for 1, 4, and 8 days, respectively. Although this "substrate cycle" has been thought of as ATP wasting, a different interpretation has recently been proposed (Issekutz,1977). This interpretation suggests that the multi-functional character of glucose-6-phosphatase is at least partly responsible for glucose cycling at the glucose-glucose-6-phosphate step, and that this is an exchange reaction which requires no energy.

The percent ^{14}C-carbon recycling suggests that a constant level of tricarbon units originally derived from glucose was again reincorporated into glucose. The calculation is the difference between replacement rate with 6-^{3}H-glucose and replacement rate with U-^{14}C-glucose, but its

significance is not entirely clear at this point due to several problems. One problem in the use of ^{14}C to estimate carbon recycling is possible dilution of the ^{14}C label by unlabelled CO_2 and acetyl CoA in the Kreb's cycle. This dilution leads to underestimates of carbon flow back into glucose (Katz et al,1976). It is also possible that $^{14}CO_2$ derived from labelled glucose may be reincorporated back into glucose, thus increasing the specific activity of glucose in the blood (Shipley and Gibbon,1975).

Levels of possible glucose precursors in vivo in the plasma also remained constant throughout the fast: lactate, pyruvate, glycerol, and alanine. In one study, plasma glycerol levels were reported to increase in chickens fasted 24 hr with no further change between 24-96 hr of fasting (Evans and Scholz,1971). However, levels of plasma precursors are often not a reliable index of turnover. Hall et al(1976) found that fasting increased glycerol production without significant changes in blood glycerol. Although fed plasma alanine levels were not obtained in this study, others have shown that these are also quite high when compared to mammals (Hill and Olsen,1963; Tasahi and Takuo,1971; Felig et al,1969).

Total blood ketone levels increased in this study between fed and fasted chickens. The significance of this in regard to fuel utilization is unclear at this point. Perhaps levels need to build up to a certain threshhold for transfer and utilization in tissues to occur.

The redox state of cellular compartments has been

estimated by the cellular lactate/pyruvate ratio(cytosol) and the β-hydroxybutyrate/acetoacetate ratio (mitochondria) (Gumaa et al,1971). The assumptions inherent in these measurements are that: 1) the metabolites reside in one compartment only or have free access across membranes such that equal distribution can occur; 2) the activity of the mediating enzyme be high enough to keep the reaction close to equilibrium; 3) the enzyme mediating the reaction must be confined to one compartment. Alterations in these ratios have been shown to be correlated with changes in PEP and glucose production in rat, rabbit, guinea pig, and pigeon (Hanson,1974; Garber and Hanson,1971 a,b; Arinze et al,1973; Soling et al, 1970).

In species which possess a cytosolic PEPCK, a reduced mitochondrial redox state is associated with increased PEP and glucose production. However, in species with predominantly mitochondrial PEPCK, like the chicken, an oxidized mitochondrial state is associated with increased PEP and glucose production (Hanson,1974; Garber and Hanson,1971 a,b; Arinze et al,1973; Soling et al,1970). The data presented here show maintenance of glucose production, but an increased β-hydroxybutyrate/acetoacetate ratio in liver and kidney in a species with mitochondrial PEPCK. This appears anomalous, but a close look at β-hydroxybutyrate dehydrogenase activity could explain these data. The activity reported in chicken liver in this study is very low. This has also been shown to be true in pigeon liver (Bailey and Horne,1972). Thus,

this pair of metabolites does not satisfy criteria for a redox metabolite indicator pair because the low enzyme activity does not fulfill assumption 2) above. On the other hand, glutamate dehydrogenase and lactate dehydrogenase showed higher activities, which allowed their use in redox estimation. The glutamate/α-ketoglutarate ratio decreased with fasting, as expected for a species with mitochondrial PEPCK. The lactate/pyruvate ratio was constant with fasting. It has been found that dietary alterations affect hepatic lactate/pyruvate ratios. In the rat, fasting increases the lactate/pyruvate ratio, while in the chick, 2 hr fasting decreased the ratio (Yeh and Leveille,1971). In this study, no further decrease occurred after 1 day of fasting.

Liver and kidney PEPCK activities were quite high in the chickens and suggested a high capacity for glucose production which was reflected in the glucose turnover rates obtained. If enzyme activities obtained for various species are compared, it appears that avian capacity is very high. Soling et al (1973) compared rat guinea pig, and pigeon total PEPCK activity and found pigeon liver enzyme activity 2 to 30 times greater depending on dietary state. Further, both rat and guinea pig cytosolic enzyme activity increased with fasting, while pigeon liver mitochondrial enzyme remained constant. In this study, chicken liver and kidney enzyme activities also remained constant at a high level. Guinea pig mitochondrial enzyme has been shown to increase with fasting in one study (Elliott and Pogson,1977).

In summary, the results show that the fasting chicken did not decrease glucose utilization per kg body weight, but did decrease glucose utilization per animal as weight loss occurred. Plasma lactate, pyruvate, glycerol, and alanine levels remained constant with fasting. Blood α-hydroxybutyrate values increased dramatically, reflecting increased production or decreased utilization and/or excretion. The differences in avian versus mammalian metabolism in fasting provide a basis for comparison under other types of nutritional alteration.

GLUCONEOGENESIS IN ISOLATED CHICKEN HEPATOCYTES

Introduction

Glucose production in avian liver has been reported to differ from that in mammals. In contrast to the rat and the guinea pig, Soling(1973) has found that isolated perfused pigeon liver does not produce glucose from pyruvate. Sarkar (1971) and Davison and Langslow (1975) have shown that,in vivo, chickens do not utilize pyruvate or alanine to increase blood glucose levels. Glycerol is converted to glucose at one-half the rate of lactate in isolated guinea pig hepatocytes (Arinze and Rowley,1975), but only at 3% the rate of lactate in chicken hepatocytes (Dickson and Langslow,1977).

The influence of β-hydroxybutyrate and fatty acids on glucose production also differs by species. β-hydroxybutyrate increases the conversion of lactate to glucose in perfused rat liver (Arinze et al,1973), but depresses that in guinea pig liver (Arinze and Rowley,1975). In the perfused rat liver, fatty acids stimulate glucose production from lactate and pyruvate (Williamson et al,1969; Soling,1974; Arinze et al,1973), but in rabbit hepatocytes and perfused guinea pig liver, octanoate inhibits the conversion of lactate to glucose and stimulates the conversion of pyruvate to glucose (Zaleski and Bryla,1977; Arinze et al,1973). In the perfused pigeon liver, fatty acids have no effect on glucose production from lactate or pyruvate (Soling and Kleineke, 1976).

These differences among species might be explained by the intracellular compartmentation of a key gluconeogenic

enzyme--phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32). In the rabbit and chick, the enzyme is primarily mitochondrial, while in the rat the enzyme is primarily cytosolic. In the guinea pig, cat, and human, the enzyme is about equally distributed in both compartments (Hanson, 1974).

Few studies have focused on the control of gluconeogenesis in species where PEPCK is located primarily in the mitochondrial compartment. Thus, it was of interest to use isolated hepatocytes from chickens to determine the conversion of various substrates to glucose and the effect of β-hydroxybutyrate and octanoate on the system.

Materials and Methods

Chemicals. Glucostat and collagenase were obtained from Worthington Biochemicals, Freehold,NJ. Octanoate-1-^{14}C and $^{3}H_2O$ were purchased from Amersham-Searle, Chicago,Ill. All other enzymes and substrates were obtained from Sigma Chemical, St. Louis, Mo.

Animals. Male broiler chicks were obtained from Fairview Farms, Remington, In, at 2 days of age and fed a commercial high carbohydrate diet (Master Mix Chick Starter, Central Soya, Ft. Wayne, In.) for 6 days, then fasted 1 day. Water was available at all times, and room lights were on 24 hr/day.

Preparation of Isolated Hepatocytes. After cervical dislocation, livers were perfused in situ with Krebs-Ringer bicarbonate buffer, pH 7.4, until most of the blood was

removed. The liver was then removed and finely minced, placed in buffer which had been bubbled with 95% O_2-5%CO_2 and contained collagenase (1 mg/ml). Flasks were incubated for 25 minutes at 41 C under 95%O_2-5%CO_2 in a Dubnoff metabolic shaker. The resulting slurry was filtered through silk mesh and centrifuged 2-3 minutes at 300xg. The cells were resuspended in oxygenated buffer and washed 3 more times before final suspension in buffer with 1.25% albumen or gelatin. Final yield was 10-20% of liver wet weight. Final suspension was about 2% cells (vol/vol) in buffer plus protein. Results were expressed per unit dry weight of cells. The dry weight of cells was determined by the difference between dry weight of cells plus buffer and the dry weight of buffer alone.

Cell viability was ascertained by the exclusion of 0.4% trypan blue dye and/or release of lactic dehydrogenase to the medium during the incubations (Bergmeyer and Bernt, 1974). The enzyme released into the medium was approximately 5% of the total cellular enzyme. Problems with detergents used in cleaning the plastic ware inhibited viability without affecting the ability to exclude dye at one point in the experiments. This led to the conclusion that dye exclusion is, at best, a crude indicator of cell viability. The problems were eliminated by acid-ethanol washing the plastic ware and the experiments continued.

Incubation. The incubations were carried out in plastic 25 ml Erlenmeyer flasks in 2.2 ml Krebs-Ringer bicarbonate

buffer containing 1.25% albumen or gelatin plus 2mM $CaCl_2$. Albumen was used in experiments in which octanoate was utilized, gelatin was used in all other experiments, as no differences were noted between them initially. All substrates were dissolved in buffer and added to the incubation media in the concentrations indicated. Flasks were gassed with O_2-CO_2 and the cells and buffer were incubated for 40 minutes at 41 C (3-5 mg dry weight). All incubations were done in triplicate for each experiment. In addition, several experiments were performed for each substrate addition such that a number of days and different animals were tested for each condition. Incubations were stopped with 0.5 ml of 36% trichloroacetic acid and neutralized with 5 M potassium carbonate. The slurry was centrifuged at 400xg for 15 minutes and the glucose in the supernatant measured by the glucose oxidase procedure. Endogenous glucose production was measured in each experiment , as well as accumulation of glucose in the presence of 10 mM lactate. Some variation in the quantitative results was found in different experiments, probably due to variation among chicks, collagenase batches, and the preparative procedures. However, no qualitative differences were observed.

The incorporation of ^3H_2O into fatty acids was measured in one experiment (Brady et al,1976). Oxidation of octanoate-1-^{14}C to $^{14}CO_2$ was measured in several experiments. The CO_2 was collected in hyamine hydroxide (Goodridge,1973). In several experiments lactate (Gutman and Wahlefeld,1974) , pyruvate (Czok and Lamprecht,1974), β -hydroxybutyrate

(Williamson and Mellanby,1974), and acetoacetate (Mellanby and Williamson,1974) were measured by standard enzymatic methods.

Statistics. Means were tested by the Student's t-test (Steel and Torrie,1960).

Results and Discussion

Glucose production from various substrates. Glucose production from lactate was linear for at least 60 minutes. The rates of conversion for different cell concentrations at each time also showed a linear response between 5-15 mg dry weight per incubation. Weight/volume adjustments were made to fit this range in all subsequent experiments. There was no lag period in glucose accumulation with lactate as substrate, as found with the rat (Cornell et al,1973) or guinea pig (Arinze and Rowley,1975). Dickson and Langslow (1977) did not observe a lag in glucose production from lactate in the chick hepatocyte either. The reasons for the observed lag in the other species could be related to a time-dependent shift in redox state or an increase in necessary metabolic intermediates (Zahlten et al,1973; Arinze and Rowley,1975). The latter authors found that NH_4Cl decreased the lag time and suggested the mechanism involved increasing glutamate, which participates in the glutamate-aspartate shuttle. This shuttle is important in species with cytosolic PEPCK. They also found that the β-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios decreased on preincubation without lactate. Thus, changes in redox state were implicated in the

lag period of the guinea pig.

The dose-response curves for glucose accumulation in the presence of lactate, pyruvate, and fructose are shown in Figure 2. For lactate, the maximal response occurred between 5 and 10 mM substrate concentration, with no further stimulation at 15 and 20 mM, similar to the guinea pig response (Arinze and Rowley,1975). Maximal fructose conversion to glucose was observable at 10-20 mM concentration. Pyruvate conversion was lower than that of lactate and fructose at all concentrations studied. At 10 mM, pyruvate conversion to glucose was only about 32% that of lactate, in contrast to the guinea pig (Arinze and Rowley,1975), rat (Story et al,1976; Veneziale et al,1976), and lamb (Clark et al,1976), where it is about equal. At 10, 15, and 20 mM fructose concentrations, glucose production from fructose was 30-50% less than from lactate, but still approximately 50-100% greater than from pyruvate. Fructose conversion to glucose has been found higher than that of lactate in the guinea pig (Arinze and Rowley,1975), rat (Story et al,1976; Veneziale et al,1976), and chickens (Dickson and Langslow,1977).

Table 12 presents the data for the conversion of various substrates to glucose by chick hepatocytes. Lactate conversion was comparable to that observed in the rat, guinea pig, and lamb hepatocytes. The relative rate of pyruvate conversion to glucose agrees well with that of isolated pigeon liver cells (Soling and Kleineke,1976) and another study of chick hepatocytes (Dickson and Langslow,1977). The reason for the

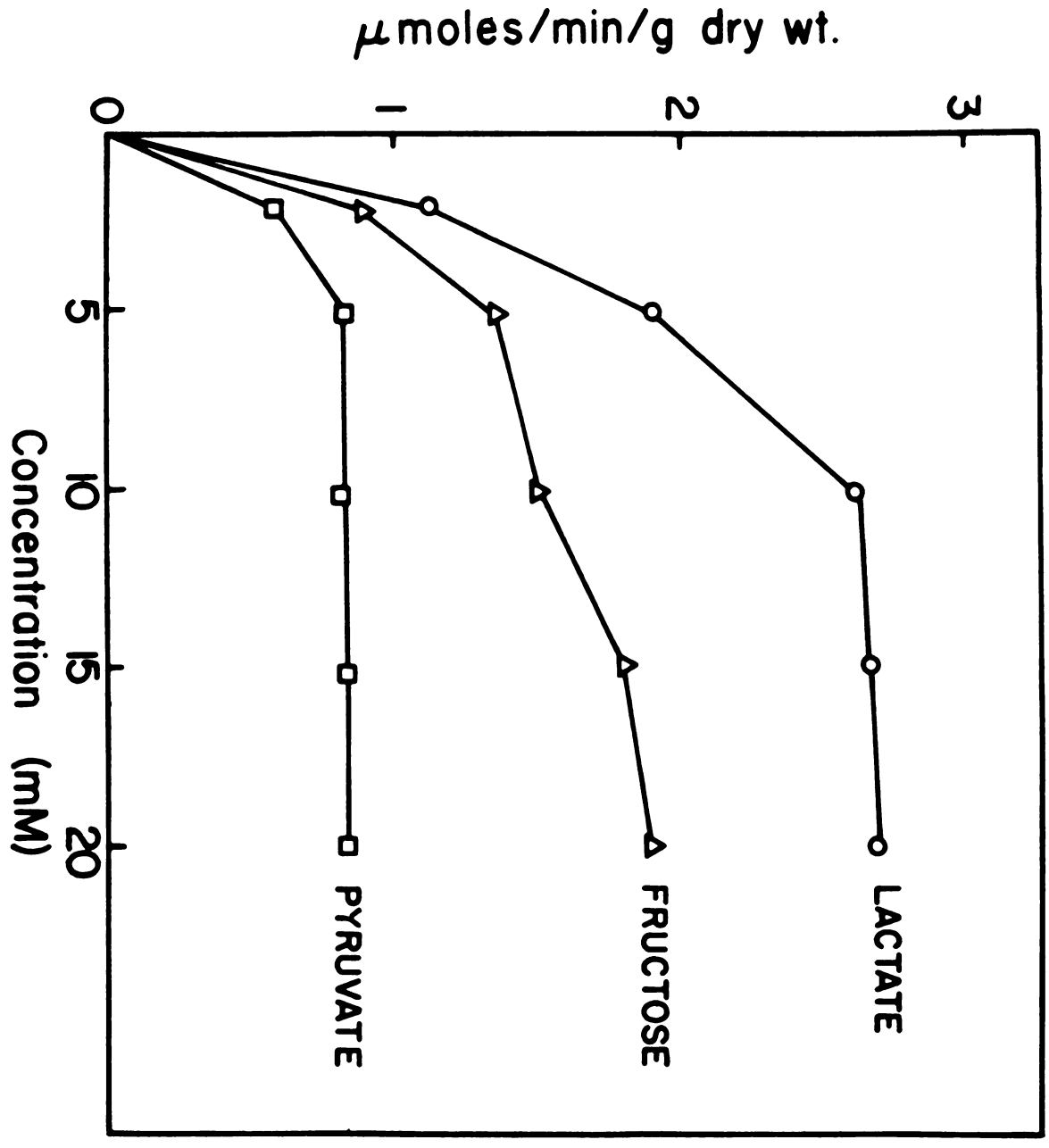

Figure 2. Conversion of lactate, pyruvate, and fructose to glucose at various substrate concentrations.

Table 12. Conversion of various substrates 10mM to glucose.

Substrate	µmoles glucose accumulated/min/g dry wt.
Lactate	2.47 ± 0.18[a]
Pyruvate	0.57 ± 0.09[b]
Fructose	1.20 ± 0.15[c]
Glycerol	0.14 ± 0.04[d]
Dihydroxyacetone	2.40 ± 0.11[a]
Glyceraldehyde	1.64 ± 0.25[c]
Malate	< 0.05
Citrate	< 0.05
Isocitrate	0.12 ± 0.08[d]
α-Ketoglutarate	< 0.05
Fumarate	< 0.05
Succinate	0.53 ± 0.20[bf]
Alanine	0.09 ± 0.05[d]
Serine	0.08 ± 0.04[d]
Glycine	< 0.05
Glutamate	< 0.05
Glutamine	< 0.05
Threonine	< 0.05
Aspartate	0.19 ± 0.09[d]
Xylitol	0.24 ± 0.04[d]
Sorbitol	0.74 ± 0.11[b]
Galactose	0.17 ± 0.10[d]
Propionate	< 0.05

Values shown represent the mean ± SEM of at least 3 experiments done in triplicate. Incubations were carried out at 41°C for 40 minutes. Endogenous values have been subtracted from each value above. For details of the experimental procedure, see Materials and Methods. Values with different superscript letters are different ($P < 0.05$).

lower conversion of fructose to glucose in the present study compared to others in unclear at this point.

Glycerol was not well utilized to form glucose, but both dihydroxyacetone and glyceraldehyde led to rates similar to lactate (Table 12). As the glycerol rates were low and those for dihydroxyacetone and glyceraldehyde relatively high, it appears that the rate limiting step in glycerol conversion to glucose occurs before glycerol enters the glycolytic pathway at the triose stage. The rate limiting step could possibly be uptake (Sestoft et al,1977) or phosphorylation (Harding et al,1975). The results for glycerol are similar to those obtained by Dickson and Langslow (1977) with isolated chicken hepatocytes. Glycerol raises blood glucose to the same extent as lactate does, when injected intraperitoneally in chickens (Davison and Langslow,1975).

Citric acid cycle (CAC) intermediates were not well utilized for glucose formation. The reasons for the low conversion to glucose may be limited permeability (Ross et al,1967; Dickson and Langslow,1977) or the lack of generation of cytosolic reducing equivalents to reverse glycolysis. Succinate was converted to glucose at the same rate as pyruvate, which may reflect its metabolism to pyruvate.

Amino acids were also poor glucose precursors. Alanine, an important glucose precursor in vivo in most species, was just as poorly utilized as other amino acids. In the perfused rat liver, alanine was 60% as effective as lactate, while in guinea pig hepatocytes, alanine rates are about

50% of lactate (Arinze et al,1973; Arinze and Rowley,1975). There are several possible explanations for the low rates observed with alanine in the present study. One may be limited permeability (Dickson and Langslow,1977); another may be the lack of reducing equivalents once alanine is converted to pyruvate. A third may be the distribution and activity of alanine aminotransferase (AAT) in the chicken. DeRosa and Swick (1975) showed that chicken liver mitochondrial AAT activity was 50-60% of mammals, while cytosolic activity was about 1%. Sarkar (1974,1977) found similar distributions. DeRosa and Swick postulated that the mitochondrial enzyme is important in converting alanine to pyruvate. Thus, the chick should be capable of converting at least 50-60% as much alanine to glucose as mammals, based on enzyme activity alone. The cytosolic alanine aminotransferase contribution to the conversion of alanine to pyruvate is not known in the chicken to this point. Thus, it is probable that a combination of these reasons, and perhaps others, are responsible for the low alanine conversion to glucose in vitro.

Sorbitol and xylitol were somewhat better glucose precursors than CAC or amino acid intermediates. This implies a functional but not very active pentose pathway (Goodridge, 1968). Other substrates tested (galactose and propionate) were not well utilized for glucose production.

Effect of Supply of Reducing Equivalents. Since it has been postulated that a lack of cytosolic reducing equivalents might be responsible for the low conversion of pyruvate to

glucose (Soling et al,1973), several substrates that produce cytosolic reducing equivalents were incubated with 10 mM pyruvate--sorbitol, xylitol, ethanol, and glycerol (Table 13). Pyruvate plus sorbitol, ethanol, or xylitol produced a greater than additive effect in glucose accumulation. The rate of conversion of pyruvate plus xylitol or sorbitol to glucose was similar to that of lactate alone. Glycerol plus pyruvate produced only a slightly greater than additive effect on glucose production, possibly again due to limited metabolism of glycerol. The results obtained for ethanol are somewhat similar to those obtained by Soling et al (1973) in perfused pigeon liver. In that system, ethanol increased glucose production from pyruvate, but did not increase it to the rates observed for lactate. In the present study, pyruvate plus sorbitol, xylitol, ethanol, or glycerol stimulated glucose production less than found by Dickson and Langslow (1977), where these substrates produced up to 3 times the additive effect. The results suggest that the lack of reducing equivalents is not the only limitation in pyruvate conversion to glucose.

Besides conversion to glucose, it is possible for pyruvate, alanine, or glycerol to be converted to fatty acids, lactate, or to be oxidized. Table 14 shows the results of experiments where cells were incubated with lactate, pyruvate, glycerol, or alanine, and the accumulation of glucose, lactate, and pyruvate measured at the end of incubation. Pyruvate was converted to lactate to a greater extent than to glucose.

Table 13. Pyruvate conversion to glucose in the presence of glycerol, xylitol, sorbitol or ethanol.

Substrate	umoles glucose accumulated/ min/g dry weight
Lactate	1.83 ± 0.25[a]
Pyruvate	0.51 ± 0.03[b]
Glycerol	0.31 ± 0.07[c]
Sorbitol	0.67 ± 0.12[b]
Xylitol	0.41 ± 0.11[bc]
Ethanol	-0.05 ± 0.01[d]
Pyruvate + glycerol	0.90 ± 0.30[b]
Pyruvate + sorbitol	1.92 ± 0.33[a]
Pyruvate + xylitol	1.30 ± 0.17[a]
Pyruvate + ethanol	0.64 ± 0.25[b]

Each value is the mean of four experiments done in triplicate. Incubations were performed as described in the Materials and Methods. All substrates were added at 10 mM. Endogenous glucose accumulation (average 0.42 ± 0.20 umoles/min/g dry weight) was subtracted from all values. Values with different letter superscripts are different ($P<0.05$).

Table 14. Glucose, lactate and pyruvate accumulation from various substrates (10mM).

Substrate	nmoles accumulated/assay		
	Glucose	Lactate	Pyruvate
Lactate	678	---	762
Pyruvate	142	557	---
Glycerol	50	91	ND
Alanine	20	74	75

Values are the mean of 2 experiments. For details see Materials and Methods. ND = not detectable. All incubations were carried out with lactate, pyruvate, glycerol or alanine as substrate. Glucose, lactate and pyruvate accumulation were measured. Endogenous accumulation of glucose, lactate and pyruvate averaged 135, 468 and 11 nmoles accumulated per assay, respectively; these values were subtracted from the accumulation observed in the presence of substrates.

This could further deplete cytosolic reducing equivalents, as NADH is needed to convert pyruvate to lactate. If the lack of cytosolic reducing equivalents is part of the reason that pyruvate is converted to glucose at very low rates, then the conversion of pyruvate to lactate would further exacerbate the situation. This conversion was also found in perfused pigeon liver (Soling et al,1973) and in isolated chicken liver cells (Ochs and Harris,1977). Glycerol was converted to lactate at a low rate, and alanine was converted to both lactate and pyruvate, as well as to glucose, at low rates. The conversion of all these substrates to fatty acids in hepatocytes from fasted chickens was low (2 to 14 nanomoles of 2-carbon units/assay). The oxidation of these substrates was not measured.

Effects of β-hydroxybutyrate and Octanoate. Since PEPCK is almost totally intramitochondrial in the chicken, and changes in mitochondrial redox state have been shown to affect the disposition of oxaloacetate (Hanson,1974; Soling, 1974; Garber and Hanson,1971 a,b), the effect of β-hydroxybutyrate on glucose production from lactate and pyruvate was also examined. It was expected that β-hydroxybutyrate would be metabolized to acetoacetate in the mitochondria via β-hydroxybutyrate dehydrogenase, thus producing mitochondrial reducing equivalents. Results in isolated guinea pig mitochondria showed that less PEP and more malate and aspartate were formed under these conditions (Garber and Hanson,1971 a,b). In species with predominantly mitochondrial PEPCK,

this could lead to decreased glucose production. However, in chicken hepatocytes, glucose production from neither lactate nor pyruvate was affected by addition of 1, 5, 10, or 20 mM β-hydroxybutyrate (Table 15). Part of the explanation for the lack of effect in avian liver is a very low activity of β-hydroxybutyrate dehydrogenase (Bailey and Horne,1969; Brady et al,1978). Thus,β-hydroxybutyrate would be converted to acetoacetate at very low rates. Indeed, we found no acetoacetate detectable in the media after incubation with β-hydroxybutyrate. This substrate has been shown to stimulate lactate conversion to glucose in rat liver (Arinze et al, 1973), but to inhibit conversion in guinea pig liver where PEPCK is 50% mitochondrial (Arinze and Rowley,1975). β-hydroxybutyrate has been found to increase pyruvate conversion to glucose in guinea pig hepatocytes (Arinze and Rowley,1975).

Octanoate, a medium chain fatty acid, has been shown to be rapidly metabolized in rat liver (Bach et al,1976; McGarry and Foster,1971; McGarry and Foster,1974; Ontko, 1972) and to produce mitochondrial reducing equivalents. In these experiments with chicken hepatocytes, the octanoate emulsion was prepared according to Spector and Steinberg (1965,1967). Octanoate had no effect on glucose production from pyruvate, but depressed that from lactate at 5 mM (Table 16). In rabbit and guinea pig liver, octanoate (1mM) stimulated glucose production from pyruvate and inhibited that from lactate. The reason for the stimulation from pyruvate might be that, in these species, cytosolic PEPCK

Table 15. Effect of β-hydroxybutyrate on glucose production from lactate and pyruvate.

Substrate	μmoles glucose accumulated/ min/g dry weight
10mM Lactate	2.77 ± 0.43
+ 1 mM βHB	2.97 ± 0.47
+ 5 mM βHB	2.73 ± 0.52
+ 10 mM βHB	2.58 ± 0.56
+ 20 mM βHB	2.67 ± 0.45
10mM Pyruvate	0.40 ± 0.08
+ 1 mM βHB	0.54 ± 0.10
+ 5 mM βHB	0.38 ± 0.07
+ 10 mM βHB	0.30 ± 0.08
+ 20 mM βHB	0.45 ± 0.10

Values are means for 5 experiments done in triplicate. For details, see Materials and Methods. All substrates were added at the concentartions indicated at the beginning of the experiment. Endogenous glucose accumulation has been subtracted from all above values. β-hydroxybutyrate addition had no effect on glucose production from either lactate or pyruvate.

Table 16. Effect of octanoic acid on glucose production in isolated chick hepatocytes.

Substrate	µmoles glucose accumulated/min/g dry wt.
Lactate	2.97 ± 0.35^{a}
Lactate + 1 mM octanoate	3.53 ± 0.42^{a}
Lactate + 5 mM octanoate	1.91 ± 0.32^{b}
Pyruvate	0.66 ± 0.10^{c}
Pyruvate + 1 mM octanoate	0.83 ± 0.18^{c}
Pyruvate + 5 mM octanoate	0.67 ± 0.08^{c}

Values are the mean of 3 experiments done in quadruplicate. Octanoate and lactate or pyruvate were added at the beginning of the experiment. For details, see Materials and Methods. Endogenous glucose accumulation was subtracted from the above values. Values with different superscript letters are different ($P < 0.05$).

increases in the fasted state (Hanson,1974; Ray,1976). If octanoate increased the mitochondrial NADH/NAD^+ ratio, more malate would be formed, which could then transfer carbon and reducing equivalents out of the mitochondria, the malate for utilization by cytosolic PEPCK after conversion to oxaloacetate. Cytosolic PEPCK activity increases under conditions of active gluconeogenesis in embryonic and young chicks (Peng et al,1973; Jo et al,1974 a,b), but perhaps its overall contribution to glucose production is minimal in chick hepatocytes.

Since the effect on lactate conversion to glucose was seen only at 5 mM octanoate, one difference among species may be in their ability to metabolize fatty acids. Soling et al,1975) have shown less uptake of fatty acids by perfused livers from fed pigeons. The production of -hydroxybutyrate during the incubation of chicken hepatocytes with octanoate was also measured in the present study, as an indication of octanoate metabolism. The amounts produced (1-10 moles/g dry wt/40 minutes) were lower than found for rabbits (Zaleski and Bryla,1977), rats (McGarry and Foster, 1974), and guinea pigs (Arinze et al,1973). $^{14}CO_2$ production from 1-^{14}C-octanoate was 10-20 µmole 1-^{14}C-octanoate converted to $^{14}CO_2$/g dry wt./40 minutes. Soling et al (1973) have found that fatty acid conversion to CO_2 decreases with fasting in both pigeons and rats.

The differences in glucose production observed in the present study may be related partly to PEPCK distribution. The results for pyruvate suggest that a lack of cytosolic

reducing equivalents is at least partially responsible for low pyruvate conversion to glucose in vitro. The results for 5 mM octanoate and lactate also suggest that, given high enough fatty acid concentrations, glucose production can be affected in this species. However, other data are not explained by PEPCK distribution. The low rate of alanine conversion to glucose may be explained by lower hepatic alanine aminotransferase activity, an intracellular distribution of this enzyme different from mammals, low permeability of alanine, or lack of reducing equivalents. The low glycerol conversion may be the result of low glycerol kinase activity or inhibition of glycerol uptake. The low conversion of citric acid cycle intermediates and amino acids may be due to limited cell or mitochondrial uptake of these substrates. The lack of effect of β-hydroxybutyrate on glucose production might be explained by the low activity of β-hydroxybutyrate dehydrogenase, thus a low conversion of β-hydroxybutyrate to acetoacetate. Therefore, the differences in glucose production in the chicken possibly result from more than one factor that is different from mammalian species.

LACTATE AND ALANINE TURNOVER IN THE CHICKEN

Introduction

Lactate and alanine are important glucose precursors in vivo for various mammalian species--man, dog, and rat (Kreisberg et al,1970; Felig,1975; Forbath and Hetenyi,1967; Belo et al,1977; Freminet et al,1975a,b; Bier et al,1977). Felig (1975) has calculated that, in postabsorptive man, alanine extraction by the liver accounts for a minimum of 6-12% of hepatic glucose output and lactate for 15-20%. Various periods of fasting have been shown to influence alanine and lactate metabolism (Freminet et al,1975 a,b; Kreisberg et al,1970; Veiga et al,1977; Felig,1975), as have various diets (Belo et al,1977). In the chicken, there is no available data on lactate or alanine turnover, although related studies have been done. Sarkar (1971) has shown that intraperitoneal alanine injection does not increase blood glucose, while Davison and Langslow (1975) have shown that it does, although at a later time than for lactate. Veiga et al (1977) have found ^{14}C alanine incorporation into glucose in the chicken increased with fasting, while that of the black vulture, a carnivorous bird, decreased.

In vitro, both alanine and lactate are converted to glucose in isolated rat hepatocytes and perfused liver. However, in avian species the results are different. Lactate is a good glucose precursor, while pyruvate is not as well converted to glucose as in the rat and guinea pig. In the chicken, alanine incorporation into glucose in vitro is negligible (Dickson and Langslow,1977; Brady et al, unpublished; Arinze and Rowley,1975; Veneziale et al,1976 a,b).

Amino acids are somewhat better glucose precursor in the rat and guinea pig than in chickens also (Dickson and Langslow,1977; Brady et al,unpublished; Arinze and Rowley,1975; Veneziale et al,1976 a,b). These observations, together with the high glucose replacement rate of chickens (Belo et al,1976; Brady et al,1977,1978), and maintenance of blood glucose during fasting (Belo et al,1976; Brady et al, 1978) pose the question of which glucose precursors are important in vivo in the chicken. Therefore, this study was designed to establish the methodology for lactate and alanine turnover studies and to begin to assess the relative conversion of lactate and alanine to glucose.

Materials and Methods

Animals. Male broiler chicks were obtained from Townline Hatcheries, Zeeland, Michigan, and fed a commercial high carbohydrate diet (Master Mix Chick Starter, Central Soya, Ft. Wayne, Indiana) until reaching 1-1.7 kg. Water was available ad libitum and room lights were on 24 hr/day.

Experiments. A series of initial experiments were performed to establish pertinent background data in the chicken. The first experiment used venous blood for both infusion and withdrawal as previous glucose turnover data had been derived via this method; the second experiment determined lactate and alanine differences in arterial and venous blood, since in other species, lactate at least, has been shown to be released from tissues in large quantities; the third series of experiments determined alanine specific activity in both

blood and plasma, since in other species the plasma appears to be the specific precursor pool for liver extraction of alanine; the fourth experiments used arterial blood or plasma for withdrawal and a vein for infusion. Wiener and Spitzer (1974) had previously found no difference in lactate specific activity in blood vs. plasma; thus, this was not tested.

In the first experiments, catheters were implanted in both jugular veins (Becton-Dickinson, Rutherford, New Jersey) After 1 hr, a priming dose of tracer was given (2 uCi lactate or alanine (U-^{14}C) plus 5 uCi 6-^{3}H glucose) (Amersham-Searle, Arlington Hts., Ill), followed by a continuous infusion of .225 uCi/min of U-^{14}C lactate or alanine and .750 uCi/min of 6-^{3}H glucose. The birds remained in good condition generally during the experiments, although in some cases the hematocrits fell with repeated sampling. Volumes withdrawn were replaced with physiological saline . The time to reach the specific activity plateau of ^{3}H-glucose and U-^{14}C alanine or lactate was approximately 150 minutes.

Arterial-venous differences in alanine and lactate were examined in the next experiments. The birds were anesthetized with 20 mg/kg sodium pentobarbital. A carotid artery was exposed and cannulated with a 23 G, 1 inch cannula (Deseret, Logan, Utah). The cannula was sutured to the surrounding muscle. A 22 G, 2 inch cannula (Becton-Dickinson, Rutherford, NJ) was implanted in the jugular vein. The skin was sutured over the cannulas and they were flushed with heparinized

saline. Samples were taken in the anesthetized and recovered state.

In the lactate and alanine turnover experiments, one carotid artery and one jugular vein were prepared as described above in 24 hour fasted chickens of approximately 1.5 kg. The infusions were given as noted above after the animals had recovered from the anesthesia (4-5 hr). Lactate, alanine, and glucose turnover were calculated according to Kreisberg et al (1970) as well as the percent of glucose arising from lactate and alanine. In the alanine infusion experiments, lactate was also isolated and the conversion of alanine to lactate was measured.

Analytical procedures. Whole blood was precipitated in 3% perchloric acid in a 1:3 ratio. The samples were neutralized with 5 M potassium carbonate, centrifuged at 2000 rpm for 5 min and the supernatants used for metabolite assays. Plasma was obtained by centrifuging the samples at 2000 rpm for 15 min, then precipitating with barium hydroxide-zinc sulfate as described previously (Belo et al,1976). The supernatant was used for metabolite assays.

Glucose was measured in plasma or whole blood by the glucose oxidase method (Worthington Biochemicals, Freehold, NJ). Lactate was determined in whole blood supernatant by the procedure of Gutman and Wohlefeld (1974) and alanine in the plasma supernatant by the method of Schutgens et al (1977).

Lactate was isolated on Dowex 1 X 8 (200-400 mesh) or

a mixture of Dowex 1X8 and Dowex 50 W (100-200 mesh), (3inch). Elution of glucose occurred in 20 ml (90-95%) of water. This washing was collected directly into scintillation vials, dried to remove $^{3}H_2O$, and counted. Various procedures were compared for the elution of lactate using 1 N HCl, acetic acid, or formic acid (VonKorff, 1969; Kreisberg et al,1970; Bartlett,1959). No differences were found among elution procedures. Lactate elution was monitored using a mixture of 6^{3}H glucose and U-^{14}C lactate with every sample run. To minimize variability and to check recovery, specific activity of the column eluate was also determined. Recovery of lactate was 60-80%. The method of Kusaka and Ui (1969) was used to test pyruvate contamination of lactate, which was found negligible.

Alanine isolation was tested by several methods. All samples were first run of Dowex 50W resin according to Harris et al (1961) to separate amino acids from other metabolites. The procedure of Hall et al (1977) was also used. Recovery by both methods was 75%. The extent of ^{14}C alanine conversion to lactate was assessed using a combination of the above methods. Although ^{14}C alanine conversion to other amino acids has been shown negligible in sheep (Wolff and Bergman (1972), the conversion was assessed in chickens als. Alanine eluted from the columns was assayed by thin layer chromatography; 90% of the ^{14}C was recovered with the alanine spot. The rest was recovered in the neighboring spots.

Results

Turnover Using Venous Blood Sampling. Chickens were infused with 6-^{3}H glucose and either U-^{14}C alanine or lactate after implantation of indwelling cannulas in the jugular veins after a 1 or 4 day fast. Since only 2 chickens were done at each time and for each tracer, statistics will not be applied. The glucose replacement rates were 18.9 and 21.0 mg/min/kg at 1 day and 16.9 and 14.2 at 4 days. These values are similar to those found for the same size chickens in other studies (Brady et al,1977; Brady et al,1978). Lactate turnover(umoles/min/kg) was 52.2 and 93.3 at 1 day and 40.7 and 83.3 at 4 days. Alanine turnover (umoles/min kg) was 13.2 and 13.6 at 1 day and 23.0 and 22.4 at 4 days.

Arterial-Venous Differences. Since lactate turnover was twice to three times that found in humans and slightly higher than found in dogs for the infusion method (Kreisberg et al, 1970; Forbath and Hetenyi,1967), venous sampling was compared with arterial sampling of blood. The reason for this is that either lactate or alanine may be released into venous blood by muscle, and thus change the apparent specific activity(Reilly and Chandrasena,1977). When lactate or alanine is released from muscle, the unlabelled metabolite will dilute the specific activity in relation to arterial blood. Thus, the estimate of turnover will be higher than if specific activity were determined on arterial blood. For lactate, the arterial values at 1 day of fasting were 1.6 umole/ml $\pm$ .2 vs. 2.1 $\pm$ .1 for venous values.

Alanine values were more variable, but averaged .84 ± .2 and .96 ± .2 for arterial and venous samples respectively.

Alanine Turnover in Arterial Blood and Plasma. Reports in the literature (Hall et al,1977; Bergman and Heitman,1978) indicate that the immediate precursor pool from which the liver extracts alanine is the plasma, not whole blood. Thus, it would be most correct to use plasma alanine specific activity if this were true in all species. If the specific activity in the red cell were lower than that in the plasma due to reaching different equilibration points, a mixture of both would lead to lower specific activity than would be obtained using plasma alone. This in turn, would lead to a higher estimation of alanine turnover, which is based on specific activity. In this study, chickens were infused with U-^{14}C alanine. The specific activities in the blood were lower than in the plasma; thus, the values estimated for turnover rate were higher for whole blood. The values obtained were: 12.0 and 12.5 in whole blood and 9.5 and 6.9 in plasma (umoles/min/kg).

Alanine and Lactate Turnover Using Arterial Sampling. Chickens weighing 1.2-1.7 kg were used in the study, as the arteries of smaller birds were not easily cannulated. As noted before (Belo et al,1976; Brady et al,1977), glucose turnover values for this size chicken using 6-^{3}H glucose were lower than for smaller animals, although a residual effect of the anesthetic may also have depressed the values in the present study. As expected, no differences were found in glucose

replacement rate with 6-^{3}H glucose, regardless of whether U-^{14}C alanine or lactate was simultaneously infused. The values were 9.9 ± .4 mg/min/kg. Plasma glucose averaged 250 ± 10 mg/dl. Lactate turnover rate was 12.1 ± .7 umole/min/kg, while alanine turnover rate was 6.9 ± .6 umole/min/kg. The percent of glucose derived from lactate was 47 ± 6; the percent of glucose derived from alanine was 8.5. The latter value was derived by assuming that the percent glucose derived from lactate would be the same in animals infused with either U-^{14}C lactate or U-^{14}C alanine, since a significant proportion of the ^{14}C alanine was converted to lactate (25-40%).

Discussion

While both lactate and alanine are considered important glucose precursors in man (Kreisberg et al,1970; Felig,1975) and dog (Belo et al,1977; Forbath and Hetenyi,1967), studies of the conversion of these metabolites to glucose in vivo in the chicken are limited. Sarkar (1971) has shown that blood glucose does not increase following intraperitoneal alanine injection, but Davison and Langslow (1975) have shown that it does. Both studies found that lactate injection increased blood glucose in vivo. Thus, the present study sought to establish lactate and alanine turnover rates in the chicken and the contribution of each to the synthesis of glucose.

The sampling site for estimating lactate and alanine

turnover is important. In the case of lactate, large amounts may be released from muscle into the venous system, thereby diluting venous specific activity and increasing the apparent turnover rate. This was confirmed in the present study, as venous blood lactate concentration was significantly higher than arterial. Lactate turnover rates based on venous blood sampling were calculated to be higher than those based on arterial sampling. Wiener and Spitzer (1974) found no differences in lactate specific activity between whole blood and plasma in dogs; because the lactate specific activity may be diluted by glycolysis, whole blood was precipitated immediately in the present study and specific activity determinations made from neutralized supernatant.

Alanine sampling in blood is also complicated by the fact that red blood cell alanine apparently does not equilibrate rapidly with plasma alanine. Thus, specific activity in whole blood is lower than in plasma. This was found to be the case in the present study. The specific activity difference becomes even more important when one considers that the liver extracts alanine from the plasma compartment (Chiasson et al,1977).

The values for lactate turnover are within the ranges for other species--rats, humans, lambs, when expressed similarly (Freminet et al,1975 a,b,1976 a,b; Prior and Christenson,1977; Kreisberg et al,1970). The percent of glucose arising from lactate was 47%, which is higher than found

for humans (Kreisberg et al,1970) where the estimate was 15-17%. In growing ewe lambs, this estimate was 20%. Thus, it appears that a greater amount of glucose is derived from lactate in the chicken. However, it should be noted that not all the experiments were performed under the same conditions.

The alanine turnover rate was similar to growing lambs, but not as high as that of fetal lambs (Prior and Christenson, 1977) or dogs (Belo et al,1977). As in the fetal lamb, appreciable amounts of ^{14}C from alanine were found in lactate, suggesting that alanine was converted to lactate. Thus, when the calculation of the percent of glucose arising from ^{14}C alanine was made, the percent contribution arising from lactate was subtracted, leading to an estimate of 8.5%. Without a correction, the percent of glucose arising from alanine would be overestimated.

The value for the percent of glucose derived from alanine is about 18% of that derived from lactate. This compares with an estimate of alanine conversion to glucose in vitro in the chicken which is 4% of lactate. Thus, it is possible in vivo that other factors contribute to a higher conversion of alanine to glucose, and the possibility of other organs such as the kidney playing a role in converting alanine to pyruvate should be considered. DeRosa and Swick (1975) have found high alanine aminotransferase activity in chicken kidney.

GENERAL CONCLUSIONS

Various studies have suggested some basic differences in glucose production between birds and mammals. The present experiments were done in order to examine glucose metabolism in the chicken in some detail and to compare the results with similar data in mammalian species which have been derived from other investigators.

Glucose replacement rate, percent recycling, mean transit time, and glucose mass were examined using various double labelled glucose tracers--2^{3}H, U^{14}C; 3^{3}H, U^{14}C; 4^{3}H, U^{14}C; 5^{3}H, U^{14}C; and 6^{3}H, U^{14}C. Estimates of replacement rate were greatest for 2^{3}H-glucose(2T), with 3T, 4T, 5T, and 6T glucose all having similar values (21.4 mg/min/kg vs. 15.8, 15.6, 17.0, and 16.0 respectively). Calculated glucose mass based on all tritiated tracers (734-1086 mg/kg body weight) agreed closely with the direct determination of body glucose (969 mg/kg). Reincorporation of tritium from $^{3}H_2O$ into glucose did not occur to any significant degree. The young chick was found to have a rapid rate of glucose turnover and high percent recycling compared to mammals.

When chickens were fasted for 1, 4, or 8 days, significant decreases occurred in total body protein and fat with fasting, the greatest energy loss from fat. Glucose production determined with either 2T or 6T glucose injected simultaneously with U-^{14}C glucose remained constant with fasting at 10-13 mg/min/kg body weight. Blood lactate and glycerol were unchanged with fasting, while pyruvate increased and plateaued. Plasma alanine, serine and glycine levels were high compared to values in fasted mammals. Blood

β-hydroxybutyrate increased dramatically with fasting (350-3500 nm/ml), while acetoacetate remained constant. The hepatic lactate/pyruvate ratio was unchanged with fasting, while the β-hydroxybutyrate/acetoacetate ratio increased. These ratios have been reported to influence phosphoenolpyruvate and glucose production in mammals. Hepatic and renal phosphoenolpyruvate carboxykinase levels remained constant, while hepatic lactate dehydrogenase increased with fasting. β-hydroxybutyrate dehydrogenase levels were very low at all times. The results indicated that little glucose sparing adaptation per kg occurs in the chicken with fasting.

Glucose production was also studied in isolated avian hepatocytes. The highest rate of glucose production was obtained from lactate, followed by dihydroxyacetone, glyceraldehyde, and fructose. Alanine was converted to glucose at only about 4% the rate of lactate, in contrast to the rat and guinea pig, where it has been found to be converted at 40-50% the rate of lactate. Pyruvate was utilized to form glucose at only 20-30% the rate of lactate, again in contrast to the rat and guinea pig, where both substrates are utilized equally well. Addition of 10 mM sorbitol, xylitol, or ethanol increased glucose production from pyruvate 25-40%, while glycerol addition increased it only 9%. Addition of β-hydroxybutyrate had no effect on glucose production from lactate or pyruvate. Addition of octanoate had no effect on glucose production from pyruvate, but depressed

it from lactate at 5 mM. Differences in the formation of glucose from various substrates suggest some basic differences in the mode of glucose production between the chick and the rat and guinea pig.

Lactate and alanine turnover and the percent glucose arising from lactate and alanine were calculated in vivo. Lactate turnover was 12.1 umole/min/kg, while alanine turnover was 6.9 umole/min/kg. The percent of glucose derived from lactate was 47%, while that from alanine was 8.5%. There was also a significant conversion of ^{14}C alanine to ^{14}C lactate. The estimate of alanine conversion to glucose in vivo is higher than estimates in vitro and may suggest that other organs may contribute to alanine metabolism and conversion to glucose. Lactate conversion to glucose was higher than in other species.

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