C H A P T E R III

Studies on some of the enzymes of carbohydrate metabolism and chemical composition of hen tibiae

CHAPTER III

STUDIES ON SOME OF THE ENZYMES OF CARBOHYDRATE METABOLISM AND CHEMICAL COMPOSITION OF HEN TIBIAE

The literature reviewed in Chapter I suggests that glycolysis, the hexose monophosphate shunt and the tricarboxylic acid cycle operate in tibiae. Most of the evidence has been obtained from studies made on cartilage slices (Dixon and Perkins, 1952; Albaum, Hirschfeld and Sobel, 1952b; Niatt and Shortt, 1953; Boyd and Neuman, 1954; Tulpule and Patwardhan, 1954; Follis and Melanotte, 1956; Dikshit, Joshi and Patwardhan, 1956; Joshi, Dikshit and Patwardhan, 1957; Meyer, et al, 1959; Whitehead and Weidmann 1959b; Borle et al, 1960a,b; Vaes and Nichols, 1961; Krishna Rao and Patwardhan, 1961). Crude homogenates and cell free extracts have also been used by some investigators, the former by Gutman and Gutman (1941). Castellani and Zambotti (1956), Dikshit (1959), Person and Fine (1959) and Cipera and Willmer (1962;63) to study phosphorylase, hexosamine synthetase, cytochrome oxidase, succinoxidase, alkaline phosphatase, ATPase and pyrophosphatase, and the latter by Dixon and Perkins (1952), Van Reen and Losee (1958), Van Reen (1959), and Hekkelman (1961) to study aconitate hydratase and NADP-isocitrate dehydrogenase.

Although the above studies present sufficient evidence for the metabolic activity of the bone, not all the important enzymes have been systematically investigated. Further, some of the studies have been made on rachitic bones and their response to vitamin D. Also not many studies have been reported on the regional distribution of these enzymes in different areas of the bone and their possible relation to degree of calcification in different regions. The few studies that have been done have not included all the areas investigated in the present study. The present studies were designed to extend our area of information in the above respects.

Studies were made of (a) the chemical composition of different regions of tibia with regard to calcium, phosphorus, nitrogen hexosamine, and citrate, and (b) the activities of certain key enzymes of glycolysis, tricarboxylic acid cycle, hexose monophosphate shunt as well as certain other enzymes. The enzymes investigated were : α-glucan phosphorylase (E.C., 2.4.1.1), lactate dehydrogenase, (E.C. 1.1.1.27), citrate synthase (E.C., 4.1.3.7) aconitate hydratase (E.C., 4.2.1.3), NADP-isocitrate dehydrogenase, (E.C., 1.1.1.42), glutamate dehydrogenase (E.C., 1.4.1.2), glutamine synthetase (E.C., 6.3.1.2), hexosamine synthetase, aspertate-2-oxoglutarate aminotransferase (E.C., 2.6.1.1), glucose-6-phosphate dehydrogenase (E.C., 1.1.1.49) and fumarate hydratase (E.C., 4.2.1.2).

MATERIALS AND METHODS

Chemicals :

The following chemicals were used in addition to those whose sources have been indicated in Chapter II :

Citric acid, sodium citrate, potassium bicarbonate, ammonium sulfate, magnesium chloride, butanol and hydroxylamine hydrochloride from the British Drug Houses Ltd., ; potassium dihydrogen phosphate, dipotassium hydrogen phosphate, trichloroacetic acid and acetic acid from E. Merck Co; adenosine monophosphate (Na-salt); glucose-1-phosphate, glycogen, acetyl coenzyme A, oxaloacetic acid, isocitric acid (Na-salt), l-malic acid, 2-oxoglutaric acid, pyridoxal phosphate, glucose-6-phosphate, adenosine triphosphate (Na-salt), sodium pyruvate and the reduced form of nicotinamide adenine dinucleotide from Sigma Chemical Co.; sodium fluoride and ferric chloride from Riedel-De Haen Ag. and tris - (hydroxymethyl) aminomethane from Nutritional Biochemicals Co.

Preparation of bone extract :

White Leghorn hens six to eight months old were used. The birds were exsanguinated by means of jugular drainage. The legs were removed immediately and packed in crushed ice. All the operations described below were carried out in a cold room at 0 to 4°C.

Tibiae were dissected out rapidly and separated from adhering muscles and connective tissues. Periosteum, cartilage (covering of epiphyseal head) and the epiphyseal heads were removed from the bone. The shaft was cut longitudinally into two halves and the bone marrow removed.

Thus five areas (as shown in Fig.7), viz., periosteum, cartilage, epiphyseal heads, cortical shaft and bone marrow were obtained. All samples except bone marrow were washed with a suitable buffer, blotted on filter paper, weighed and used for the preparation of enzyme extracts.

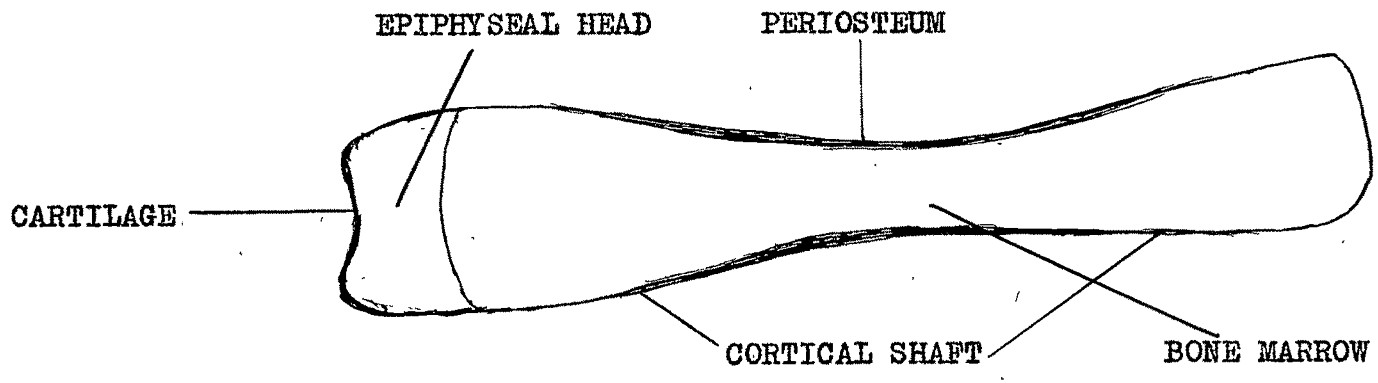

Fig. 7. Diagram showing the different areas of hen tibia.

Each area was cut into small pieces, then ground with the appropriate grinding medium for 10 minutes in a chilled mortar surrounded by a salt-ice mixture. The grinding media were: (a) 3 volumes of potassium phosphate buffer, 0.02M, pH 7.0 for the preparation of enzyme extract for the estimation of lactate dehydrogenase, citrate synthase, aconitate hydratase, NADP-isocitrate dehydrogenase, glutamate dehydrogenase, aspartate-2-oxoglutarate aminotransferase, glucose-6-phosphate dehydrogenase and fumarate hydratase; (b) 10 volumes of citric acid-sodium citrate buffer, 0.1M, pH 5.8, containing 0.15% adenosine monophosphate and 0.5% sodium fluoride for the preparation of enzyme extract for the estimation of α-glucan phosphorylase; (c) 3 volumes of citric acid sodium citrate buffer, 0.1M pH 6.5, containing 0.146% glutamine for the preparation of enzyme extract for the estimation of hexosamine synthetase; (d) 3 volumes of 0.02M potassium bicarbonate solution for the preparation of enzyme extract for the estimation of glutamine synthetase.

The homogenates were centrifuged at 9230 x g. at 0°C for 15 minutes and the supernatant used directly for the determination of the various enzyme activities.

Preliminary experiments were carried out to derive the optimum conditions with respect to the concentrations of enzyme and substrate, period of incubation and pH on the enzyme activity using (a) epiphyseal extracts for citrate synthetase, aconitate hydratase, NADP-isocitrate dehydrogenase, and hexosamine synthetase; (b) cartilage extract for glucose-6-phosphate dehydrogenase and fumarate hydratase; (c) cortical shaft extract for α -glucan phosphorylase, aspartate-2-oxoglutarate aminotransferase and lactate dehydrogenase; and (d) bone marrow extract for glutamine synthetase.

Attempt was made to detect the activity of glutamate dehydrogenase in all the areas.

The details of the assay systems, the derived optimum conditions, and procedures used in the estimation of enzyme activities are summarized in Table 10. The data establishing optimum concentrations of enzyme extract and substrate, pH and period of incubation are presented in Tables 11 to 20.

Assay components were added in (a) test tubes kept in ice for α -glucan phosphorylase, citrate synthetase, hexosamine synthetase, aspartate-2-oxoglutarate aminotransferase and (b) Beckman spectrophotometer quartz cells (1 cm light path) for lactate dehydrogenase, glutamate dehydrogenase, aconitate hydratase, NADP-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and fumarate hydratase.

For comparison of the activities of enzymes in different areas of the tibia, one adult hen was taken for each experiment and both tibiae were combined. Three experiments were conducted and the mean value calculated as enzyme units per gm wet tissue.

Protein content (N x 6.25) of the enzyme extract was estimated by the microkjeldahl method (Hawk, Oser and Summerson, 1954).

In all cases, an enzymes unit was defined as the amount of enzyme required for disappearance of one micromole of substrate or formation of one micromole of the product in 60 minutes under the assay conditions.

Except where otherwise specified, a Klett-Summerson Photoelectric Colorimeter was used for measurement of color.

<u>Determination of Chemical Composition</u> :

Five areas of tibia, viz., periosteum, cartilage, epiphyseal head, cortical shaft and bone marrow were separated as described and were defatted and dried by the method of Baker <u>et al</u> (1946). The fragments of each area were weighed after separation and then kept in a mixture of equal parts of ether and 97% alcohol at 3 to 5°C for two days. The defatted material was washed with a fresh mixture of the solvents and then dried in an air oven at 100°C for about twelve hours until constant weight was obtained. Approximately 5 to 7 mg of the dried materral from each area was weighed accurately on Mettler semimicrobalance and used for the determination of calcium and phosphorus. Nitrogen, total hexosamine and citric acid were estimated in separate portions of the dried samples. Citric acid being estimated only in cartilage, epiphyseal head and cortical shaft. Details of the estimation of different constituents have been described already in Chapter II.

TABLE - 10

Details of Enzyme Assays

Details of assay system	α-glucan phosphorylase (E.C.,2.4.1.1)	Lactate dehydrogenase (E.C., 1.1.1.27)
Reference	Cipera and Willmer (1962)	Kornberg (1955)
Buffer	citric acid-sodium citrate buffer, pH 5.8, 100 micromoles.	potassium phosphate buffer, pH 7.4, 100 micromoles
Substrate	glucose-1-phosphate, 6 micromoles.	sodium pyruvate, 5 micromoles
Amount of enzyme extract.	0.6 ml	0.5 ml
Other components.	glycogen (15 mg/ml), 0.2 ml adenosine monophosphate, 4.1 micromoles, sodium fluoride, 120 micromoles; final volume 2.6 ml.	$NADPH_2$, 0.2 micromoles; final volumes 3.0 ml.
Temperature and period of incubation.	30°C; 1 hours	30°C; 1.5 minutes
Initiation of reaction.	addition of enzyme extract	addition of enzyme extract
Termination of reaction	2.0 ml of 10% TCA (Trichloro-acetic acid) added and supernatant recovered after centrifugation.	-----
Modification for blank	enzyme added after incubation	sodium pyruvate omitted.
Parameter measured.	liberation of micromoles of inorganic phosphorus in the supernatant (Fiske and SubbaRow, 1925).	oxidation of micromoles of $NADPH_2$ measured by changes in optical density at 340 mµ at 15 seconds intervals.

Table 10 continued

Details of assay system	Citrate synthase (E.C., 4.1.3.7)	Aconitate hydratase (E.C., 4.2.1.3)
Reference	Stern et al (1951)	Racker (1950)
Buffer	potassium phosphate buffer, pH 7.2, 20 micromoles	potassium phosphate buffer, pH 7.0, 20 micromoles.
Substrate	acetyl coenzyme A, 0.5 micromoles; oxalocetate, 20 micromoles	sodium citrate, 10 micromoles
Amount of enzyme extract.	0.5 ml	0.2 ml
Other components	1-cysteine hydrochloride (neutralized), 10 micromoles; magnesium chloride 10 micromoles; final volumes 1.5 ml	Final volume 3.0 ml.
Temperature and period of incubation	30°C; 2 hours	30°C; 4 minutes.
Initiation of reaction	addition of enzyme extract	addition of sodium citrate.
Termination of reaction.	heated in boiling water bath with loose corks for 5 minutes and cooled on ice. 0.5 ml of 20% TCA added and supernatant recovered after centrifugation.	---
Modification for blank	enzyme added after incubation	citrate omitted.
Parameter measured	formation of micromoles of citrate in superinatant, measured by the method of Natelson, et al (1948)	formation of micromoles of cis-aconitate measured by changes in optical density at 240 mμ at 1 minute intervals.

Table 10 continued

Details of assay system	Isocitrate dehydrogenase (E.C., 1.1.1.42)	Glutamate dehydrogenase (E.C., 1.4.1.2)
Reference	Ramakrishnan and Martin (1955)	Bulen (1956)
Buffer	tris-HCl buffer, pH 7.5, 10 micromoles	tris-Hcl buffer, pH 8.0, 100 micromoles
Substrate	sodium isocitrate, 0.5 micromoles	2-oxoglutarate, 20 micromoles
Amount of enzyme extract	0.1 ml	0.2 ml
Other components	magnesium chloride, 10 micromoles; NADP, 0.1 micromole; final volume 3.0 ml	$NADH_2$, 0.1 micromole; ammonium sulphate, 300 micromoles; final volume 3.0 ml
Temperature and period of incubation.	30°C; 4 minutes	30°C; 2 minutes
Initiation of reaction	addition of sodium isocitrate	addition of $NADH_2$
Termination of reaction	---	---
Modification for blank.	isocitrate omitted	2-oxoglutarate omitted.
Parameter measured	formation of micromoles of $NADPH_2$ measured by changes in optical density at 340 mμ at 1 minute intervals.	oxidation of micromoles of $NADH_2$ measured by changes in optical density at 340 mμ at 30 second intervals.

Table 10 continued

Details of assay system	Glutamine synthetase (E.C., 6.3.1.2)	Hexosamine synthetase
Reference	Gothoskar, et al (1960)	Castellani and Zambotti (1956)
Buffer	tris-HCl buffer, pH 7.6, 10 micromoles.	citric acid-sodium citrate buffer pH 6.5 100 micromoles.
Substrate	glutamate, 40 micromoles	glucose-6-phosphate, 20 micromoles; l-glutamine, 10.0 micromoles.
Amount of enzyme extract	0.2 ml	0.1 ml
Other components	ATP(Na salt), 10 micromoles; $MgSO_4$, 20 micromoles; cysteine hydrochloride(neutralized), 10 micromoles; hydroxylamine hydrochloride (neutralized), 100 micromoles; final volume 2.0 ml	final volume, 2.0 ml
Temperature of period of incubation.	50°C; 1 hour	30°C; 3 hours
Initiation of reaction	addition of glutamate	additionof glucose-6-phosphate
Termination of reaction	0.8 ml of ferric chloride reagent (the reagent contained equal volumes of 10% $FeCl_3 6H_2O$ in 0.2N HCl, 24% TCA and 50% v/v HCl) added and the precipitated protein centrifuged off.	heated in boiling water bath with loose cork for 10 minutes and cooled in ice. 2.0 ml of NHCl added and supernatant recovered after centrifugation.
Modification for blank	glutamate omitted	glucose-6-phosphate added after incubation
Parameter measured	formation of micromoles of glutamyl hydroxamic acid (GHA) measured by colour intensity of ferric-hydroxymate complex at 540 mμ)	formation of micromoles of hexosamine in supernatant (Rondle and Morgan, 1955)

Table 10 continued

Details of assay system	Aspararate-2-oxoglutarate aminotransferase (E.C., 2.6.1.1)	Glucose-6-phosphate dehydrogenase (E.C., 1.1.1.49)
Reference	Shah and Ramakrishnan (1963)	Kornberg and Horecker (1955)
Buffer	potassium phosphate buffer, pH 7.0, 50 micromoles	tris-HCl buffer, pH 7.6, 100 micromoles
Substrate	l-aspartate, 10 micromoles; 2-oxoglutarate, 10 micromoles	Glucose-6-phosphate, 5 micromoles.
Amount of enzyme extract	0.2 ml	0.2 ml
Other components	pyridoxal phosphate, 0,02 micromoles; final volume, 1.0 ml	$MgCl_2$, 10 micromoles; NADP, 0.1 micromoles; final volume, 3.0 ml.
Temperature and period of incubation	37°C; 1 hour	30°C; 9 minutes
Initiation of reaction	addition of l-aspartate	addition of glucose-6-phosphate
Termination of reaction	heated in boiling water bath with loose cork for 3 minutes and cooled on ice. 1 ml. of 95% ethanol added and supernatant recovered after centrifugation.	-
Modification for blank	enzyme added after incubation	glucose-6-phosphate omitted
Parameter measured	micromoles of glutamic acid formed assayed chromatographically (Giri et al, 1953) using butanol: water: acetic acid (40:7:5) as solvent system.	formation of micromoles of $NADPH_2$ measured by changes in optical density at 340 mμ at 3 minutes intervals.

Table 10 continued

Details of assay system	Fumarate hydratase (E.C., 4.2.1.2)
Reference	Racker (1950)
Buffer	potassium phosphate buffer, pH 7.0, 20 micromoles.
Substrate	1-malate, 10 micromoles
Amount of enzyme extract	0.2 ml
Other components.	final volume, 3.0 ml
Temperature and period of incubation	30°C; 5 minutes.
Initiation of reaction	addition of 1-malate
Termination of reaction	---
Modification of blank	1-malate omitted
Parameter measured	formation of micromoles of fumarate measured by changes in optical density at 240 mμ at 1 minute intervals.

RESULTS AND DISCUSSION

The dependence of enzyme activity on enzyme and substrate concentrations, period of incubation, pH etc. for each assay are shown in Tables 11 to 20. The activity of each enzyme increased linearly with enzyme and substrate concentration and period of incubation. The pH optima derived in the present studies were similar to those for corresponding enzymes in other animal tissues.

Data on the chemical composition of tibia on dry weight basis are given in Table 21. Calcium, phosphorus, and hexosamine contents of cartilage, epiphyseal head and cortical shaft were found to be greater than those of periosteum and bone marrow, whereas nitrogen content was found to be less. Cortical shaft contained atleast twice as much of calcium and phosphorus as other regions on wet weight basis. The areas with higher calcium and phosphorus contents, viz., the cortical shaft, epiphyseal head and cartilage also showed higher Ca/P and Ca/N ratios.

A more positive relation between hexosamine content and degree of calcification was found when the data were considered on a wet weight basis.

The nitrogen content of areas with greater calcification,

Table 11

Effect of enzyme concentration, period of incubation, glucose-1-phosphate and pH on α-glucan phosphorylase activity in cell free extract obtained from the cortical shaft of hen tibiae

Variable	Inorganic phosphorus liberated (micromoles)
Enzyme (ml)**	
0.0	0.00
0.2	0.07
0.4	0.13
0.6*	0.22
0.8	0.24
Period of incubation (minutes)	
0	0.00
15	0.06
30	0.11
60*	0.26
120	0.33
Glucose-1-phosphate (micromoles)	
0.0	0.00
3.0	0.13
6.0*	0.24
12.0	0.35
15.0	0.33
pH (citric acid-sodium citrate buffer)	
5.0	0.13
5.2	0.18
5.8*	0.26
6.2	0.22
6.6	0.15

* Assay system was the same as given in Table 10 except for the variable mentioned.

** Protein content, 1.4 mg/ml.

Table 12

Effect of enzyme concentration, period of incubation and sodium pyruvate on lactate dehydrogenase activity in cell free extract obtained from the cortical shaft of hen tibia.

Variable	$NADH_2$ oxidized (micromoles)
Enzyme (ml)**	
0.00	0.000
0.25	0.022
0.50*	0.044
0.75	0.057
1.00	0.066
Period of incubation (seconds)	
30	0.015
45	0.022
60	0.029
75	0.036
90*	0.044
105	0.050
120	0.056
180	0.065
Sodium pyruvate (micromoles)	
0.0	0.000
2.0	0.019
5.0*	0.044
7.0	0.046
10.0	0.046

* Assay system was the same as given in Table 10 except for the variable mentioned.

** Original extract was diluted ten times with 0.02M phosphate buffer, pH 7.0 and then used. Protein content (original extract), 1.3 mg/ml.

Table 13

Effect of enzyme concentration, period of incubation, added oxaloacetate, added acetyl coenzyme A and pH on citrate synthase activity in cell free extract obtained from the epiphyseal head of hen tibia.

Variable	Citrate formed (micromoles)
Enzyme (ml)**	
0.0	0.00
0.2	0.08
0.5*	0.22
1.0	0.26
Period of incubation (minutes)	
0	0.00
30	0.04
60	0.09
120*	0.21
180	0.22
Oxaloacetate (micromoles)	
0	0.00
10	0.10
20*	0.22
40	0.36
60	0.38
Acetyl coenzyme A (micromoles)	
0.00	0.00
0.25	0.10
0.50*	0.20
1.00	0.36
1.50	0.38
pH (phosphate buffer for pH 6.0 to 7.5, and tris-HCl buffer for pH 8.0 and 9.0)	
6.0	0.08
7.0	0.18
7.2*	0.20
7.5	0.20
8.0	0.16
9.0	0.06

* Assay system was the same as given in Table 10 except for the variables mentioned.

** Protein content, 2.4 mg/ml.

Table 14

Effect of enzyme concentration, period of incubation, citrate concentration and pH on aconitate hydratase activity in cell free extract obtained from epiphyseal head of hen tibia

Variable	cis-aconitate formed (micromoles)
Enzyme (ml)**	
0.00	0.000
0.05	0.005
0.10	0.013
0.20*	0.026
0.40	0.045
Period of incubation (minutes)	
0	0.000
1	0.007
2	0.014
3	0.020
4*	0.028
6	0.035
Citrate (micromoles)	
0	0.000
5	0.016
10*	0.027
15	0.035
20	0.037
pH (phosphate buffer for pH 5.5 to 7.5, tris-HCl buffer for pH 8.0)	
6.0	0.013
6.5	0.016
7.0*	0.027
7.5	0.024
8.0	0.016

* Assay system was the same as given in Table 10 except for the variables mentioned.

** Protein content, 2.4 mg/ml.

Table 15

Effect of enzyme concentration, period of incubation, isocitrate concentration and pH on NADP-isocitrate dehydrogenase activity in cell free extract obtained from epiphyseal head of hen tibia

Variable	$NADPH_2$ formed (micromoles)
Enzyme (ml)**	
0.00	0.000
0.05	0.022
0.10*	0.044
0.15	0.055
Period of incubation (minutes)	
0	0.000
1	0.010
2	0.021
3	0.032
4*	0.042
6	0.051
9	0.059
Isocitrate (micromoles)	
0.00	0.000
0.12	0.018
0.25	0.036
0.50*	0.042
0.75	0.047
pH (phosphate buffer for pH 6.0 to 7.0 and tris-HCl buffer for pH 7.2 to 9.0)	
6.0	0.004
6.5	0.004
7.0	0.015
7.2	0.036
7.5*	0.044
8.0	0.040
9.0	0.022

* Assay system was the same as given in Table 10 except for the variables mentioned.

** Protein content, 2.1 mg/ml.

Table 16

Effect of enzyme concentration, period of incubation, glutamate concentration and pH on glutamine synthetase activity in cell free extract obtained from bone marrow of hen tibia

Variable	Glutamyl hydroxymate formed (micromoles)
Enzyme (ml)**	
0.0	0.00
0.1	0.19
0.2*	0.38
0.4	0.51
Period of incubation (minutes)	
0	0.00
15	0.10
30	0.17
60*	0.36
90	0.45
Glutamate (micromoles)	
0	0.00
10	0.08
20	0.16
40*	0.36
60	0.39
pH (phosphate buffer for pH 6.0 to 7.0 and tris-HCl buffer for pH 7.2 to 8.5).	
6.0	0.09
7.0	0.23
7.2	0.28
7.6*	0.37
8.0	0.34
8.5	0.14

* Assay system was the same as given in Table 10 except for the variables mentioned.

** Protein content, 3.8 mg/ml.

Table 17

Effect of enzyme concentration, period of incubation, glucose-6-phosphate concentration and pH on hexosamine synthetase activity in cell free extract obtained from epiphyseal head of hen tibia.

Variable	Hexosamine formed (micromoles)
Enzyme (ml)**	
0.00	0.000
0.05	0.083
0.10*	0.156
0.15	0.203
0.20	0.250
Period of incubation (hours)	
0	0.000
1	0.042
2	0.107
3*	0.156
4	0.179
Glucose-6-phosphate (micromoles)	
0	0.000
10	0.089
20*	0.167
30	0.185
40	0.191
pH (citric acid-sodium citrate buffer for pH 5.0 to 6.8 and tris-HCl for pH 7.0 and 8.0).	
5.0	0.030
5.5	0.072
6.0	0.143
6.5*	0.169
6.8	0.169
7.0	0.137
7.5	0.018

* Assay system was the same as given in Table 10 except for the variable mentioned.

** Protein content, 2.5 mg/ml.

Table 18

Effect of enzyme concentration, period of incubation, 2-oxoglutarate concentration and pH on aspartate -2-oxoglutarate aminotransferase activity in cell free extract obtained from the cortical shaft of hen tibia [illegible]

Variable	Glutamic acid formed (micromoles)
Enzyme (ml)**	
0.0	0.00
0.1	0.12
0.2*	0.22
0.3	0.27
0.4	0.31
Period of incubation (minutes)	
0	0.00
15	0.04
30	0.10
60*	0.22
90	0.34
120	0.37
2-oxoglutarate (micromoles)	
0	0.00
5	0.09
10*	0.22
15	0.29
20	0.32
pH (phosphate buffer for pH 6.0 to 7.0 and tris-HCl buffer for pH 7.5 and 8.0)	
6.0	0.11
6.5	0.20
7.0*	0.23
7.5	0.22
8.0	0.17

* Assay system was the same as given in Table 10 except for the variable mentioned.

** Protein content, 1.9 mg/ml.

Table 19

Effect of enzyme concentration, period of incubation and glucose-6-phosphate concentration on glucose-6-phosphate dehydrogenase activity in cell free extract obtained from the cartilage of hen tibia.

Variable	$NADPH_2$ formed (micromoles)
Enzyme (ml)**	
0.0	0.000
0.1	0.016
0.2*	0.033
0.3	0.038
0.4	0.041
Period of incubation (minutes)	
0	0.000
3	0.011
6	0.020
9*	0.032
12	0.039
Glucose-6-phosphate (micromoles)	
0.0	0.000
2.5	0.016
5.0*	0.033
7.5	0.035

* Assay system was the same as given in Table 10 except for the variable mentioned.

** Protein content, 2.3 mg/ml.

Table 20

Effect of enzyme concentration, period of incubation, l-malate concentration and pH on fumarate hydratase activity in cell free extract obtained from cartilage of hen tibia

Variable	Fumarate formed (micromoles)
Enzyme (ml)**	
0.0	0.000
0.1	0.031
0.2*	0.069
0.3	0.087
Period of incubation (minutes)	
0	0.000
1	0.014
2	0.029
3	0.044
4	0.060
5*	0.075
7	0.090
l-malate (micromoles)	
0.0	0.000
2.5	0.019
5.0	0.038
10.0*	0.075
15.0	0.094
pH (phosphate buffer for pH 5.5 to 7.5 and tris-HCl buffer for pH 8.0).	
5.5	0.012
6.0	0.019
6.5	0.050
7.0*	0.075
7.5	0.069
8.0	0.025

* Assay system was the same as given in Table 10 except for the variable mentioned.

** Protein content, 2.3 mg/ml.

TABLE - 21

Data on the chemical composition of different areas of the tibia from hen.*

Area	Dry weight	Calcium	Phosphorus	Nitrogen	Ca/P	Ca/N	Total hexosamine	Citric acid
Periosteum	31.6** (32.5, 30.5,31.7)	7.54 (7.14, 7.35,8.12)	7.12 (6.88, 7.01,7.46)	14.34 (13.36. 14.41,15.25)	1.06***	0.53***	0.45 (0.35, 0.42,0.59)	-
Cartilage	31.4 (32.1, 31.0,31.3)	24.62 (23.24, 24,51,26.12)	13.74 (13.10, 13.87,14.24)	5.31 (5.00, 5.21,5.72)	1.79	4.64	5.88 (5.32, 5.83,6.49)	0.15 (0.12, 0.15,0.19)
Epiphyseal head	46.4 (45.0, 46.6,47.6)	24.13 (23.30, 23.94,25.14)	13.94 (13.69, 14.00,14.12)	5.58 (5.04, 5.50.6.20)	1.73	4.32	5.55 (5.08, 5.46,6.12)	0.33 (0.28, 0.32,0.39)
Cortical shaft	81.8 (82.2, 81.9,81.4)	27.39 (26.60, 27.45,28.14)	14.76 (14.32, 14.86,15.11)	4.45 (4.44, 4.61,4.34)	1.86	6.14	3.39 (3.01, 3.29,3.86)	0.57 (0.51, 0.57,0.62)
Bone marrow	19.4 (18.6, 19.9,19.5)	5.57 (4.98, 5.60,6.12)	3.56 (3.04, 3.44,4.21)	8.53 (8.14, 8.52,8.93)	1.56	0.64	1.22 (1.03, 1.46,1.18)	

* Results are expressed as per cent value on dry weight basis.

** Mean value of three separate experiments. The results of each experiment are given in parentheses.

*** Results are calculated from the mean values of calcium, phosphorus and nitrogen.

viz., cortical shaft, epiphyseal head and cartilage, was less than those with less calcification, viz., periosteum and bone marrow, when the data were considered on a dry weight basis. The picture was somewhat different on a wet weight basis because of the low moisture content of cortical shaft and high moisture content of bone marrow. The Ca/N ratio was found to vary inversely with Ca/P ratio.

Among the areas for which citric acid content was determined, there appeared to be some relation between citric acid content and Ca/P ratio. Also, when the values were considered on a wet weight basis, the citric acid content of these areas viz., cortical shaft, epiphyseal head and cartilage which was 4.6, 1.5 and 0.5 mg per g respectively of fresh tissues with their calcium content 224.2, 108.4 and 74.6 mg per g respectively of fresh tissue.

The activities of the enzymes studied in different areas of tibia are given in Table 22.

The activity of lactate dehydrogenase was found to be of a much higher order than that of the other enzymes studied. This is consistent with the report of Borle <u>et al</u> (1960a) that 84% of glucose is converted to lactic acid by metaphyseal slices of adult mice. It is also interesting

TABLE - 22

Distribution of carbohydrate metabolism enzymes in different areas of hen tibia.*

Enzymes	periosteum	cartilage	epiphyseal head	cortical shaft	bone marrow
α-glucan phosphorylase	3.7** (3.1,3.7,4.2)***	1.6 (1.1,1.5,2.2)	1.9 (2.6,1.8,1.5)	4.1 (3.7,4.3,4.4)	3.6 (2.6,3.3,4.8)
Lactate dehydrogenase	1308 (1178,1339,1408)	228 (203,232,249)	309 (288,311,327)	115 (106,111,131)	545 (458,556,620)
Citrate synthase	0.8 (0.6,0.9,0.9)	0.7 (0.8,0.6,0.6)	0.7 (0.8,0.6,0.7)	0.5 (0.6,0.5,0.5)	0.6 (0.6,0.6,0.5)
Aconitate hydratase	0.4 (5.4,6.6,7.2)	5.0 (4.8,4.2,6.0)	6.2 (6.6,6.1,6.0)	1.4 (1.2,1.2,1.8)	7.0 (6.0,7.2,7.9)
NADP-isocitrate dehydrogenase	15.6 (13,1,15.6,18.0)	20.8 (19.8,19.6,22.9)	41.2 (41.9,45.8,36.0)	9.1 (11.9,9.8,6.5)	41.9 (39.2,40.8,45.8)
Glutamine synthetase	0	0	0	0	5.7 (5.1,5.4,6.6)
Hexosamine synthetase	0.7 (0.6,0.8,0.8)	0.6 (0.5,0.7,0.6)	1.7 (1.5,1.7,2.0)	1.3 (1.1,1.2,1.5)	1.1 (0.9,1.1,1.4)
Aspartate-2-oxoglutrate aminotransferase	6.9 (5.4,7.2,8.0)	2.1 (1.8,2.0,2.5)	6.8 (6.5,6.9,7.1)	3.2 (2.8,3.3,3.4)	3.2 (3.0,3.0,3.6)
Glucose-6-phosphate dehydrogenase	2.9 (2.7,3.0,3.1)	3.2 (2.7,3.3,3.5)	2.8 (2.5,2.7,3.2)	2.9 (2.4,3.0,3.3)	8.3 (7.3,8.7,8.9)
Fumarate hydratase	15.4 (12.4,14.6,19.1)	12.9 (10.1,13.4,15.1)	19.9 (19.1,19.3,21.4)	5.9 (5.1,6.2,6.5)	23.1 (22.5,22.5,24.2)

* Results are expressed as units per gm of fresh tissue.

** Mean value for fresh tissue from three hens.

*** Values for tissue from three individual hens are given in parentheses.

to note that the activity of this enzyme is less in areas showing greater calcification.

Although the activity of citrate synthase is the least in cortical shaft when considered on a wet weight basis, it is the highest when considered on a dry weight basis. The activities of aconitate hydratase and NADP-isocitrate dehydrogenase are the lowest in this region whether considered on a wet weight or dry weight basis. Although the enzyme activities are not necessarily measures of the rate at which the reactions proceed <u>in vivo</u>, particularly with low activities of the order found in the present studies, the enzyme pattern suggests a decreased breakdown of citrate in cortical shaft which is the region having the highest citrate content.

Glutamine, synthetase was found to be absent in regions other than the bone marrow. This enzyme was found to be present in hen heart tissue in previous and present studies in this laboratory. This difference would appear to account for the requirement of glutamine by chick embryonic tibiae, cultivated <u>in vitro</u> (Biggers, Gwatkin and Heyner, 1961) and its non-requirement by chick embryonic heart fibroblasts cultivated <u>in vitro</u> (Patel, Rajlakshmi and Ramakrishnan, 1965).

The presence in bone of glucose-6-phosphate dehydrogenase is to be expected on the basis of the observation (Lucy, Webb and Biggers, 1961) that the addition of labelled glucose to the culture medium was followed by radioactivity in ribose in bone rudiments.

The presence of fumarate hydratase in bone tissue has not been reported by other investigators but is to be expected on the basis of the evidence for the operation of tricarboxylic acid cycle (Whitehead and Weidmann 1959b).

The presence of aspartate-2-oxoglutarate aminotransferase provides additional evidence for the capacity of bone tissue to metabolise glucose to form amino acid.

Hexosamine synthetase was found in all the regions studied. However, as glutamine synthetase was not present in regions other than bone marrow, the glutamine required for synthesis of hexosamine must presumably come from either bone marrow or the general pool via the blood stream.

No consistent relation was found between the hexosamine content of different regions and the activity of hexosamine synthetase. Somewhat similar observations were made by Dikshit (1959) who found that the increase in hexosamine synthetase activity of rachitic bone treated with vitamin D was not associated with an increase in hexosamine content.

The low activity of the TCA cycle enzymes is consistent with the greater conversion of glucose to lactate in bone tissue reported by Borle et al (1960a). In the case of citrate synthase, aconitate hydratase, and NADP-isocitrate dehydrogenase, comparative value on bone, liver and kidney obtained by Dixon and Perkins (1952) show a greater activity of these enzymes in kidney and liver (in that order) than in bone.

The low activities of aconitate hydratase and NADP-isocitrate dehydrogenase in cortical shaft as compared to other regions are in accord with similar observations made by Van Reen and his associate (Van Reen and Losee, 1958; Van Reen, 1959) on femur of young and old rabbits and dogs. The values obtained in the present study are shown along with those obtained by Van Reen and his associate in the case of young and old rabbits and dogs femora in Table 23.

A generally greater activity of many enzymes was observed in bone marrow as compared to other regions. Glutamate

TABLE - 23

Comparison of the activities of certain enzymes obtained in present study with those of other investigations

Enzyme	Reference	Species	Regions studied	Activity in Shaft	Activity in Other regions
Aconitate hydratase	Present study	hen tibia	periosteum cartilage epiphyseal head cortical shaft bone marrow	1.4	6.2 - 7.0
	Van Reen (1959)	young rabbit femur	epiphyseal line cartilage metaphysis cortex marrow	0.41	6.60 - 12.55
		Young dog femur	epiphyseal line cartilage metaphysis cortex marrow	0.58	3.60 - 6.51
NADP-isocitrate dehydrogenase	Present study	hen tibia	periosteum cartilage epiphyseal head cortical shaft bone marrow	9.1	15.6 - 41.9
	Van Reen and Losee (1958)	growing rabbit femur	epiphysis spongiosa shaft marrow	9.0	58.0 - 115.0
	Van Reen (1959)	young rabbit femur	epiphysis metaphysis cortex marrow	9.6	76.8 - 115.3
		young dog femur	epiphysis metaphysis cortex marrow	5.4	21.0 - 48.5

dehydrogenase was not detected in any of the areas studied. It is suspected that the extraction procedure either failed to solubilize the enzyme or destroyed the enzyme. This is being investigated further.

In summary, the data obtained showed a differential distribution of calcium, phosphorus, nitrogen, and hexosamine in different regions of tibia. Some of these differences were found to be associated with those in calcification. The activities of the enzymes studied were also found to differ in different regions. The area showing the greatest calcification, viz., the cortical shaft was also found to have lower activities of certain enzymes.

SUMMARY

The different regions of tibia, viz., periosteum, cartilage, epiphyseal head, cortical shaft and bone marrow of White Leghorn hen were studied for chemical composition with regard to calcium, phosphorus, nitrogen, hexosamine and moisture. Three of the regions viz., cartilage, epiphyseal head and cortical shaft were also analysed for citric acid content. Calcium, phosphorus and hexosamine contents of cortical shaft, cartilage and epiphyseal head were higher than those of periosteum and bone marrow,

whereas nitrogen content of the former was less. Cortical shaft, cartilage and epiphyseal head showed a greater degree of calcification as judged by Ca/P and Ca/N ratios.

Studies were made on the distribution of key enzymes of glycolysis, tricarboxylic acid cycle, hexose monophosphate shunt and certain other enzymes in the above regions of tibia. The enzymes studied were: α-glucan phosphorylase, lactate dehydrogenase, citrate synthase, aconitate hydratase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, fumarate hydratase, glutamine synthetase, hexosamine synthetase and aspartate-2-oxoglutarate aminotransferase.

The activity of lactate dehydrogenase was found to be of a much higher order than that of the other enzymes studied in all the regions strengthing the hypothesis of a greater degree of glycolysis in bone.

Glutamine synthetase was found in bone marrow but not in the other areas studied.

The activities of aconitate hydratase, NADP-isocitrate dehydrogenase, fumarate hydratase and lactate dehydrogenase were lower in cortical shaft than in other regions of the bone.

In general bone marrow was found to be enzymically more active than other regions.