C H A P T E R IV

Comparative data on activities of enzymes in chick embryonic tibiae grown *in vitro* and *in vivo*

CHAPTER IV

COMPARATIVE DATA ON ACTIVITIES OF ENZYMES IN CHICK EMBRYONIC TIBIAE GROWN IN VITRO AND IN VIVO

Studies detailed in Chapter II showed that chick embryonic tibiae cultivated *in vitro* in a chemically defined medium (M 858) achieved increase in length, wet and dry weights, calcium, phosphorus, nitrogen and hexosamine. In this Chapter comparative data were also presented on tibiae of chick embryo grown *in vivo*.

Studies detailed in Chapter III showed the presence and distribution of key enzymes of glycolysis, tricarboxylic acid cycle and hexose monophosphate shunt as well as certain other enzymes in different regions of hen tibia. The results obtained suggested a broad relation between chemical composition and enzyme make up of different regions.

The question arises as to if and how the activities of these enzymes are affected during *in vitro* cultivation and the present studies were designed to answer this question. For this purpose the activities of these enzymes in 10-day-old chick embryonic tibiae cultivated *in vitro* for 4 days were compared with those in tibiae of 10-day-old

and 14-day-old chick embryos.

The data obtained were considered in the light of those obtained on chemical composition in Chapter II.

The enzymes studied were the same as in Chapter III except for the omission of glutamate dehydrogenase, fumarate hydratase and lactate dehydrogenase.

MATERIALS AND METHODS

Chemicals :

The sources of chemicals used have been described in Chapters II and III.

Preparation of cell free extracts for enzyme study :

Tibiae from 10- and 14-day-old chick embryos and 10-day-old chick embryos cultivated for 4 days *in vitro* were washed with cold physiological saline solution and blotted on filter paper. They were weighed quickly on the Mettler semimicrobalance, transferred to a previously chilled mortar, and ground for 10 minutes in a cold room with three volumes of cold grinding medium. Details of the grinding media for assay of the different enzymes have been described in Chapter III.

For the preparation of cell free extract 26 bones

from 10-day-old embryos, 16 bones from 14-day-old embryos and 36 bones from 10-day-old embryos cultivated for 4 days *in vitro* were taken for each enzyme. After grinding the bones with the appropriate buffer, the sides of the mortar were washed twice using nine volumes of the buffer each time. The homogenate and washings were combined together and centrifuged at 4000 x g. at 0°C for 15 minutes. The supernatant was used as the enzyme source. Enzyme activity was determined according to the method described in Chapter III.

RESULTS AND DISCUSSION

Data on the chemical composition of tibiae grown *in vitro* and *in vivo* for periods corresponding to those used in the present studies on enzymes are reproduced from the data of Chapter II in Table 24.

The increments in length, weight and bone constituents under the two conditions are compared in Table 25.

The increments obtained *in vivo* were larger than those obtained *in vitro* for all parameters measured, as might be expected. The increments *in vivo* were 2.5 times those *in vitro* in the case of length, 10 times in the case

Table 24

Growth and chemical composition of embryonic chick tibiae grown in vitro and in vivo*

	Tibia from chick embryos		10-day-old embryonic tibia cultivated in vitro for four days
	10-day-old	14-day-old	
	values per bone		
Length (mm)	7.5	15.1	10.5
Wet weight (mg)	3.6	31.3	6.8
Dry weight (mg)	0.40	3.54	0.72
Calcium (µg)	9.8	210.4	22.5
Phosphorus (µg)	7.7	132.6	12.6
Nitrogen (µg)	11.7	240.0	20.9
Ca/P	1.27	1.65	1.78
Ca/N	0.84	0.88	1.08
Citrate (µg)	1.06	10.8	0.00
Hexosamine (µg)	12.0	197.0	21.8

* Mean values reproduced from Tables 1, 2, 3 and 6 in chapter II. The values for 10-day-old tibiae correspond to 0-day in these tables.

Table 25

Comparative data on increase in length and other constituents of chick embryonic tibiae grown in vivo and in vitro*

	Increase during growth		Increment in vivo
	In vivo	In vitro	Increment in vitro
Length (mm)	7.6	3.0	2.5
Wet weight (mg)	27.7	3.2	9
Dry weight (mg)	3.14	0.32	10
Calcium (µg)	200.6	12.7	15
Phosphorus (µg)	124.9	4.9	25
Nitrogen (µg)	228.3	9.2	25
Citric acid (µg)	9.74	-1.06	-
Hexosamine (µg)	185.0	9.8	19
Increment in Ca / Increment in P	1.61	2.63	
Increment in Ca / Increment in P	0.88	1.55	
Increment as percentage of increment in dry weight for Calcium	6.4	4.0	
Phosphorus	4.0	1.5	
Nitrogen	7.3	2.6	

* Calculated from data of Table 24.

in the case of dry weight, and 15 to 25 times in the case of bone constituents studied suggesting that growth as measured by increase in length was much less affected by *in vitro* cultivation than the percentage composition of important constituents.

The *in vitro* cultures showed a complete disappearance of citrate which increased in tibiae grown *in vivo*. This might possibly have been due to the absence of citrate in the culture medium.

The increments in calcium and phosphorus expressed as percentage increase in dry weight were 1.61 and 2.63 for the *in vivo* and *in vitro* conditions. This suggests that calcium was deposited more rapidly than phosphorus *in vitro* than *in vivo* presumably due to the deposition of calcium in the form of salts other than phosphates. Similarly, the deposition of calcium *in vitro* would also appear to have proceeded at a relatively faster rate than that of nitrogen.

Comparative data on activities of enzymes in embryonic tibiae grown *in vitro* and *in vivo* are presented in Tables 26 and 27. The most significant observation was that the cultures were enzymically active after 4 days of cultivation *in vitro*.

In absolute terms the activities of enzymes increased both *in vivo* and *in vitro* but the increases were greater

TABLE - 26

Activities of enzymes in embryonic chick tibiae grown in vitro and in vivo

Enzyme	Tibiae from chick embryos		10-day-old embryonic chick tibiae, cultivated in vitro for four days.
	10-day-old	14-day-old	
	Units per 10 tibiae		
α-glucan phosphorylase	0.33* (0.32,0.34)**	2.49 (2.21,2.77)	0.95 (0.86,1.04)
Citrate synthase	0.25 (0.27,0.24)	0.98 (0.90,1.07)	0.39 (0.37,0.42)
Aconitate hydratase	1.59 (1.23,1.86)	7.0 (6.0 ,8.0)	2.00 (1.78,2.22)
NADP-isocitrate dehydrogenase	3.33 (2.90,3.77)	9.59 (8.19,11.0)	6.33 (6.00,6.66)
Glutamine synthetase	0	0	0
Hexosamine synthetase	0.20 (0.18,0.23)	0.93 (0.82,1.04)	0.43 (0.41,0.46)
Aspartate-2-oxoglutarate aminotransferase	0.19 (0.17,0.21)	1.30 (1.23,1.37)	0.45 (0.43,0.47)
Glucose-6-phosphate dehydrogenase	0.28	1.45	0.75

* Mean value of two separate experiments.
** The values for individual experiments are given in parentheses.

TABLE - 27

Activities of enzymes of embryonic chick tibiae grown in vitro and in vivo

Enzyme	Tibiae from chick embryos		10-day-old embryonic tibiae cultivated in vitro for four days.
	10-day-old	14-day-old	
	Units per gram of wet weight		
∝-glucan phospharylase	7.58 (8.50,6.66)	11.11 (10.18,12.04)	14.14 (12.28, 16.00)
Citrate synthase	6.21 (6.49, 5.94)	3.50 (3.31, 3.70)	4.87 (4.66, 5.06)
Aconitate hydratase	32.26 (30.77, 35.76)	26.41 (22.02,30.80)	29.31 (26.90,31.73)
NADP-isocitrate dehydrogenase	79.13 (76.31,81.96)	38.08 (42.32,33.84)	92.36 (90.93,93.80)
Glutamine synthetase	0	0	0
Hexosamine synthetase	4.75 (4.50, 5.00)	3.46 (3.85, 3.07)	6.43 (6.57, 6.30)
Asphartate-2-oxoglutarate aminotransferase	4.98 (4.72,5.25)	4.72 (4.55,4.89)	6.08 (6.03,6.14)
Glucose-6-phosphate dehydrogenase	6.67	5.41	9.48

in vivo (Table 26). It will be recalled, however, that the increase in weight *in vivo* was about 10 times that *in vitro* (Table 25) and when this difference was taken into account and the data were considered on the basis of units per gram of wet weight, the values for the *in vitro* condition were found to be greater than those for the *in vivo* condition as can be seen from Table 27. The increases in enzyme activity during growth *in vitro* and *in vivo* are compared in Table 28.

Glutamine synthetase showed no activity in tibiae grown either *in vitro* or *in vivo*. This is to be expected on the basis of results presented in the previous chapter.

The low activity of the citrate synthase as compared to the much higher activities of aconitate hydratase and NADP-isocitrate dehydrogenase might account for the disappearance of citrate during *in vitro* cultivation. The same pattern was seen in tibiae grown *in vivo* , but some citrate could have come from the general pool via the blood stream.

Again no consistant relation was observed between hexosamine content and the activity of hexosamine synthetase.

TABLE - 28

Comparative increase in enzyme activity in chick embryonic tibiae grown in vitro and in vivo*

Enzyme	Increase in activity units per 10 tibiae		Increment in vivo / Increment in vitro
	in vivo	in vitro	
α-glucan phosphorylase	2.16	0.62	3.5
Citrate Synthase	0.73	0.14	5.2
Aconitate hydratase	5.41	0.41	13.2
NADP-isocitrate dehydrogenase	6.26	3.00	2.1
Glutamine synthetase	0.00	0.00	-
Hexosamine synthetase	0.71	0.21	3.4
Aspartate-2-oxoglutarate aminotransferase	1.11	0.24	4.6
Glucose-6-phosphate dehydrogenase	1.17	0.47	2.5

* Data from Table 26 were used for calculating the increase

During *in vitro* cultivation the increase in enzyme activities corresponded to that in nitrogen content. But during *in vivo* growth enzyme activities increased only 3-7 times whereas nitrogen content increased twenty fold. This observation together with the difference in the percentage of extractable nitrogen shows that the form in which nitrogen was deposited *in vitro* was not the same as the one in which it was deposited *in vivo*.

In 2 or 3 experiments the extractable protein of the bone was determined using phosphate buffer extracts. The pecentage of the same was found to be 81% in tibiae of 10-day-old embryo, 43% in tibiae of 14-day-old embryo and 64% in the former cultivated *in vitro* for four days.

These results suggest that embryonic tibiae cultivated *in vitro* in a chemically defined medium can not only achieve growth and calcification but also maintain their metabolic activity. This observation is believed to be of considerable value as it underlines the potentialities of the technique used for further studies on bone metabolism under conditions free of host influences.

SUMMARY

Comparative data were obtained on the activities of

enzymes of glycolysis, tricarboxylic acid cycle and hexose monophosphate shunt as well as certain other enzymes ~~were~~ determined in 10-day-old and 14-day-old chick embryonic tibiae and 10-day-old chick embryonic tibiae cultivated *in vitro* in medium 858 for 4 days.

On a wet weight basis the *in vitro* cultures were found to show at least as much enzyme activity as tibiae grown *in vivo*. The data obtained were compared with those on chemical composition and with previous results where appropriate. The results underline the validity and suitability of the technique used for further studies on bone metabolism.