The Effect of Thymus Extracts on Phosphorus Compounds in Muscle and Serum, and on Serum Calcium

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1. Thymectomy in young rabbits decreased the ATP content and increased the inorganic phosphate content of skeletal muscle. The serum calcium content was decreased, whereas the inorganic phosphate content was increased. 2. The administration of a lipid fraction (TL) or protein fractions (CIF and TP) of thymus extracts to thymectomized rabbits in short-term experiments increased the ATP content of muscle and decreased the inorganic phosphate contents of muscle and serum. Serum calcium content was increased. 3. The action of the thymus extract TP was specific only on the phosphate compounds, since the increase in serum calcium concentration was also caused by the control extract from muscle. The action of the extract TL is not specific, being paralleled by the action of a control extract from muscle.

The thymus gland has been studied intensively, but its role in metabolism is still not clear. Parhon & Costin (1953) found a decrease in the ATP and ADP contents of muscle after thymectomy. Parhon & Apostol (1954, 1956) and Parhon (1955) found that the protein fraction of a thymus extract stimulated the respiration of muscle *in vitro*.

Bomskov (1940) and Bomskov, Kaulla & Maurath (1941) investigated a lipid extract from the thymus and found that it had hyperglycaemic activity. Milcu & Apostol (1950) have also shown that the thymus influences the metabolism of glucose in muscle. They found that a protein fraction from thymus extracts had hyperglycaemic activity and anti-insulin activity when tested *in vitro* with diaphragm muscle. Pora & Toma (1960b) found that an aqueous extract of the thymus (CIF) increased the glucose metabolism of poisoned and fatigued frog gastrocnemius.

In the present paper we report our studies on the ATP and inorganic phosphate contents of skeletal muscle (femoral biceps muscle) and the inorganic phosphate and calcium contents of serum in thymectomized rabbits treated with various thymus extracts.

MATERIALS AND METHODS

Animals. Prepubescent rabbits weighing 400–500 g. were used. Some were thymectomized and killed between 4 and 28 days after operation. Control animals were untreated. Groups of thymectomized animals were treated with thymus extracts. One group of control animals was used for sham operation.

Thymus extracts. Three different preparations from the

thymus were used. The first was an aqueous extract (fraction CIF) containing protein, prepared by the Industrial Pharmaceutical Centre, Bucharest. The other two were a polypeptide-containing fraction (TP) and a lipid-containing fraction (TL) prepared in the laboratory. Fractions from calf skeletal muscle (MP and ML) were prepared in the same way as TP and TL respectively (see below) as controls.

Preparation of fractions TP and TL. Fresh calf thymus was cleaned and frozen and the broken material was extracted with several changes of cold acetone for 72 hr. The residue was dried *in vacuo* and extracted three times with anhydrous ether. Acetone and ether were removed by distillation and the residues were combined and dissolved in chloroform; the solution was filtered and chloroform removed from the filtrate. The residue (fraction TL) was dissolved in olive oil, 1.0 ml. corresponding to 1.0 g. of fresh thymus gland. The extract was prepared in the cold, avoiding contact with air when possible, and stored under nitrogen.

Fraction TP was obtained from the acetone- and ether-extracted residue remaining from the preparation of fraction TL as follows: 100 g. of powder was extracted with 100 ml. of 0.1 N-NaOH for 24 hr. The preparation was diluted and the residue removed. The supernatant was diluted and warmed to 70°; 0.1 N-HCl was added to give pH 4–5. The precipitate that formed was removed by filtration and 0.1 N-NaOH was added to the filtrate to give pH 10. After a further filtration the filtrate was adjusted to pH 7 with 0.1 N-HCl.

Injection of thymus extracts. Short-term and long-term experiments were carried out. In short-term experiments, animals 4 days after thymectomy were killed 2–24 hr. after a single intraperitoneal injection of extract equivalent to 2 g. of fresh thymus/100 g. body wt. In long-term experiments, animals were injected with extract equivalent to 0.5 g. of fresh thymus/100 g. body wt. on alternate days for 28 days beginning 4 days after thymectomy. When the

Table 1. *ATP concentrations in muscle of thymectomized rabbits before and after injection of thymus and control extracts (short-term experiment)*

Experimental details are given in the text. Values are expressed as mg. of ATP P/100g. of muscle, and are given as means $\pm$ s.e.m. with the numbers of observations in parentheses. In this and subsequent Tables the statistical interpretation was calculated from the following formulae:

$$\sigma_1 = \sqrt{\frac{\sum d_1^2}{n(n-1)}}; \sigma_2 = \sqrt{\frac{\sum d_2^2}{n(n-1)}}; \tau = \sqrt{\sigma_1 + \sigma_2}$$

$\bar{R}_1$ and $\bar{R}_2$ are the means of the controls and treated animals. When $\bar{R}_1 - \bar{R}_2 \geq 2\tau$, the difference is significant (Sig.); when $\bar{R}_1 - \bar{R}_2 < 2\tau$, the difference is not significant (Not sig.).

Control animals	Thymec- tomized	Thymectomized + fraction CIF				Thymectomized + fraction TP				Thymectomized + fraction TL		Thymec- tomized + fraction MP
		2hr.	4hr.	8hr.	24hr.	2hr.	4hr.	8hr.	24hr.	4hr.	8hr.	
23-2	11-10	20-2	24-2	22-0	25-2	21-9	25-7	21-9	23-3	23-2	25-1	13-2
$\pm 1-17$	$\pm 0-85$	$\pm 1-33$	$\pm 1-76$	$\pm 1-34$	$\pm 1-77$	$\pm 1-34$	$\pm 0-50$	$\pm 0-20$	$\pm 2-32$	$\pm 3-20$	$\pm 1-30$	$\pm 3-80$
(50)	(40)	(30)	(15)	(10)	(10)	(15)	(15)	(10)	(15)	(15)	(10)	(10)
	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Not sig.

Table 4. *Inorganic phosphate concentrations in serum of thymectomized rabbits before and after injection of thymus and control extracts (short-term experiment)*

Experimental details are given in the text. Values are expressed as mg. of P/100ml. of serum, and are given as means $\pm$ s.e.m. with the numbers of observations in parentheses.

Control animals	Thymec- tomized	Thymectomized + fraction CIF				Thymectomized + fraction TP				Thymectomized + fraction TL				Thymectomized + fraction MP	
		2hr.	4hr.	8hr.	12hr.	2hr.	4hr.	8hr.	2hr.	4hr.	8hr.	2hr.	4hr.	8hr.	
8-3	10-0	7-4	7-3	8-3	7-3	7-4	9-4	8-2	8-6	8-8	8-2	9-4	10-5	10-3	
$\pm 0-12$	$\pm 0-23$	$\pm 0-23$	$\pm 0-22$	$\pm 0-36$	$\pm 0-12$	$\pm 0-24$	$\pm 0-52$	$\pm 0-67$	$\pm 0-09$	$\pm 0-35$	$\pm 0-60$	$\pm 0-10$	$\pm 0-17$	$\pm 0-19$	
(50)	(40)	(30)	(15)	(10)	(10)	(15)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	
	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Not sig.	Not sig.	Not sig.	Sig.	Not sig.	Not sig.	Not sig.	

animals had been killed, samples of blood and skeletal muscle (femoral biceps muscle) were obtained. The concentrations of ATP and inorganic phosphate in muscle and the concentrations of inorganic phosphate and calcium in serum were estimated.

In the extracts as finally prepared 1 ml. corresponded to 1 g. of thymus or muscle. Fraction TL is a complex lipid-containing extract and contained 77mg. of total lipids/ml.; the lipid-containing muscle fraction (ML) contained 50mg. of total lipids/ml. The chemical and chromatographic analyses of fraction TL showed the presence of free fatty acids, glycerides, steroids, phospholipids and cerebrosides (Potop, Boeru & Mreană, 1963a). The analysis of fractions TP and CIF showed on electrophoresis in the Tiselius apparatus the presence of a single fraction, which was a polypeptide. Chromatographic analysis showed the

presence of 16 free amino acids (Potop, Boeru & Mreană, 1963b). The proteinaceous extracts contained a quantity of total N that corresponded for fraction TP to 1.19mg., for fraction CIF to 0.57mg. and for fraction MP to 1.43mg./ml. of extract.

Analytical methods. ATP was estimated by the method of Gurvici, Leviant & Ervina (1950). The ATP was precipitated from the trichloroacetic acid supernatant as the mercury salt; the increase in inorganic P after hydrolysis for 10min. with N-HCl was then determined. The results (ATP P) are expressed as mg. of acid-labile P/100g. of muscle.

Calcium was estimated by the method of Clark & Collip (1925).

For inorganic phosphate estimation the method of Briggs (1922) was used.

RESULTS

Table 2. *ATP concentrations in muscle of thymectomized rabbits before and after injection of thymus and control extracts (long-term experiment)*

Experimental details are given in the text. Values are expressed as mg. of ATP P/100g. of muscle, and are given as means $\pm$ S.E.M. with the numbers of observations in parentheses.

Control animals	Thymectomized	Thymectomized + fraction CIF	Thymectomized + fraction MP
26.2 $\pm$ 2.83 (20)	26.7 $\pm$ 0.91 (20) Not sig.	32.7 $\pm$ 2.39 (20) Sig.	29.3 $\pm$ 1.20 (10) Not sig.

A short-term experiment (see Table 8) with sham-operated as well as normal control animals showed no significant effect of the sham operation on the values studied in this work. All other experiments were done with normal animals as controls.

ATP in muscle. The results given in Table 1 show that in short-term experiments the ATP content of muscle decreased 52% after thymectomy, and that injection of thymus extracts CIF, TP and TL gave significant increases 2hr. after injection of 81.8, 97.2 and 109.9% respectively of the values for thymectomized animals. Fraction MP had no

Table 3. *Inorganic phosphate concentration in muscle of thymectomized rabbits before and after injection of thymus and control extracts (short-term and long-term experiments)*

Experimental details are given in the text. Values are expressed as mg. of P/100g. of muscle, and are given as means $\pm$ S.E.M. with the numbers of observations in parentheses.

Short-term experiment			Long-term experiment				
Control animals	Thymectomized	Thymectomized + fraction CIF	Control animals	Thymectomized	Thymectomized + fraction CIF	Thymectomized + fraction TP	Thymectomized + fraction MP
65.2 ± 2.60 (50)	79.9 ± 2.69 (40) Sig.	71.9 ± 1.22 (30) Sig.	66.1 ± 2.24 (20)	68.0 ± 2.60 (20) Not sig.	64.5 ± 2.65 (20) Not sig.	64.9 ± 5.09 (15) Not sig.	65.0 ± 1.80 (10) Not sig.

Table 5. *Inorganic phosphate concentrations in serum of thymectomized rabbits before and after injection of thymus and control extracts (long-term experiment)*

Experimental details are given in the text. Values are expressed as mg. of P/100ml. of serum, and are given as means $\pm$ S.E.M. with the numbers of observations in parentheses.

Control animals	Thymectomized	Thymectomized + fraction CIF	Thymectomized + fraction TP	Thymectomized + fraction TL	Thymectomized + fraction MP
8.2 $\pm$ 0.12 (20)	8.4 $\pm$ 0.23 (20) Not sig.	9.0 $\pm$ 0.28 (20) Sig.	9.2 $\pm$ 0.17 (20) Sig.	10.0 $\pm$ 0.11 (10) Sig.	8.5 $\pm$ 0.28 (10) Not sig.

Table 6. *Calcium concentrations in serum of thymectomized rabbits before and after injection of thymus and control extracts (short-term experiment)*

Experimental details are given in the text. Values are expressed as mg. of Ca/100ml. of serum, and are given as means $\pm$ s.e.m. with the numbers of observations in parentheses.													
Control animals	Thymec-tomized	Thymectomized + fraction C1F						Thymectomized + fraction TP					
		2hr.	4hr.	8hr.	24hr.	2hr.	4hr.	8hr.	2hr.	4hr.	8hr.	2hr.	4hr.
12-4	10-0	12-8	11-5	11-5	10-7	11-1	10-1	7-6	11-7	11-3	9-8	12-7	11-8
± 0.21	± 0.16	± 0.19	± 0.16	± 0.19	± 0.15	± 0.21	± 0.08	± 0.14	± 0.20	± 0.12	± 0.20	± 0.05	± 0.28
(70)	(70)	(70)	(15)	(10)	(10)	(15)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.

Table 8. *ATP and inorganic phosphate concentrations in muscle and inorganic phosphate and calcium concentrations in serum of thymectomized rabbits before and after injection of thymus and control extracts (short-term experiment : 2 hr.)*

Experimental details are given in the text. Values are expressed as means $\pm$ s.e.m. of eight observations.

ATP in muscle (mg. of ATP P/100g.)	Control animals	Sham-thymec-tomized	Thymec-tomized	Thymec-tomized + fraction TP		Thymec-tomized + fraction TP		Control animals	Thymec-tomized + fraction TL		Thymec-tomized + fraction ML
				25-8 $\pm$ 0.72	17-9 $\pm$ 1.44	66-6 $\pm$ 1.33	73-2 $\pm$ 3.31	33-3 $\pm$ 2.60	34-3 $\pm$ 0.33	31-0 $\pm$ 0.2	30-6 $\pm$ 1.72
	25-0 $\pm$ 1.44	25-8 $\pm$ 0.72	17-9 $\pm$ 1.44	Not sig.	Sig.	Not sig.	Not sig.	33-3 $\pm$ 2.60	Not sig.	31-0 $\pm$ 0.2	Not sig.
P _i in muscle (mg. of P/100g.)	62-3 $\pm$ 2.33	62-6 $\pm$ 1.03	66-6 $\pm$ 1.33	Not sig.	Not sig.	76-5 $\pm$ 2.50	70-0 $\pm$ 2.00	70-0 $\pm$ 2.00	94-0 $\pm$ 4.33	94-0 $\pm$ 4.33	90-4 $\pm$ 2.48
P _i in serum (mg. of P/100ml.)	7-53 $\pm$ 0.18	7-40 $\pm$ 0.05	8-35 $\pm$ 0.42	Not sig.	Sig.	9-70 $\pm$ 0.20	9-95 $\pm$ 0.05	9-00 $\pm$ 0.18	8-26 $\pm$ 0.14	8-84 $\pm$ 0.37	Not sig.
Calcium in serum (mg. of Ca/100ml.)	11-3 $\pm$ 0.066	11-6 $\pm$ 0.42	9-55 $\pm$ 0.26	Not sig.	Sig.	9-50 $\pm$ 0.36	9-42 $\pm$ 0.18	11-2 $\pm$ 0.20	11-3 $\pm$ 0.28	11-3 $\pm$ 0.18	Not sig.

Table 7. *Calcium concentrations in serum of thymectomized rabbits before and after injection of thymus and control extracts (long-term experiment)*

Experimental details are given in the text. Values are expressed as mg. of Ca/100 ml. of serum, and are given as means $\pm$ S.E.M. with the numbers of observations in parentheses.

Control animals	Thymectomized	Thymectomized + fraction CIF	Thymectomized + fraction TP	Thymectomized + fraction TL	Thymectomized + fraction MP
12.5 $\pm$ 0.21 (20)	10.6 $\pm$ 0.32 (20) Sig.	11.6 $\pm$ 0.54 (20) Sig.	12.4 $\pm$ 0.13 (20) Sig.	13.0 $\pm$ 0.23 (10) Sig.	13.0 $\pm$ 0.19 (10) Sig.

significant effect, but in another experiment (see Table 8) fraction ML showed as great an effect as fraction TL. The effect of fraction TP, but not that of fraction TL, may thus be regarded as specific. It was found (Table 2) that 28 days after thymectomy the ATP concentration had returned to its normal value, perhaps by replacement of thymus function by the lymphatic system. The injection of fraction CIF in long-term experiments caused an increase of 22.4% in ATP concentration. Fraction MP also produced a 9% increase in ATP concentration, but this is not statistically significant.

Inorganic phosphate in muscle. The concentration of inorganic phosphate in muscle (see Table 4) increased significantly by 22% after thymectomy in short-term experiments, and the injection of fraction CIF decreased it by 11%. In another short-term experiment (see Table 8) fraction TL caused an increase in inorganic phosphate concentration, but this effect was not specific, being paralleled by that of the muscle extract ML. In long-term experiments (Table 3) the inorganic phosphate concentration increased by only 2.8%, which is not significant. Injection of fraction CIF, TP or MP had no significant effect.

Inorganic phosphate in serum. The concentration of inorganic phosphate in serum in short-term experiments increased significantly by 20% after thymectomy, as shown in Table 4. Injection of thymus extracts decreased the phosphate concentration: fraction CIF decreased it by 26%, fraction TP by 27% and fraction TL by 18% 2 hr. after injection. Fraction MP had no effect up to 8 hr. after injection. Fraction ML caused a significant decrease after 2 hr. (see Table 8). Table 5 gives the results of the long-term experiments. The value for inorganic phosphate after thymectomy was the same as that of the control. Injection of fraction CIF, TP or TL increased the inorganic phosphate concentration but the control extract MP had no effect. The specificity of the effect of thymus proteinaceous extracts is thus shown in both types of experiment, but that of the lipid-containing extract is not specific.

Calcium in serum. The concentration of serum calcium was decreased both 4 and 28 days after thymectomy (Tables 6 and 7). In the short-term experiment, the thymus extract CIF was more effective than fraction TP or TL in raising the calcium concentration: the effects were greatest at 2 hr., and fell off thereafter. In long-term experiments also the thymus extracts were effective in raising the calcium concentration. It is to be emphasized that the administration of MP control extract also produced a significant increase in serum calcium concentration in both types of experiment. Thus the effect of TP extracts on serum calcium concentration is not specific, as is the effect on the phosphorus compounds studied. The effects of fractions TL and ML in short-term experiments were the same (Table 8), and thus no specific thymus effect is found here either.

Tests in a short-term experiment with boiled TP extract gave results not significantly different from those given by the unboiled extract (Table 8).

DISCUSSION

Our experiments demonstrate that the thymus influences the metabolism of phosphorus and calcium, and has some effect on the ATP metabolism of skeletal muscle. Much evidence already mentioned had earlier been accumulated that the thymus can affect respiration and carbohydrate metabolism. The changes in the ATP content of muscle might be explained by stimulation of its synthesis or by a diminution in turnover and increase in 'storage ATP' (Hohorat, Reim & Bartels, 1962).

Szent-Györgyi (1957) considers the lymphatic system and the thymus together in their effect on neuromuscular activity and suggests that there is very possibly a link between a hormonal activity of the thymo-lymphatic system and the disease of myotony and muscular dystrophy.

The results showing that thymectomy decreases serum calcium concentration and increases serum inorganic phosphate concentration (in short-term experiments) may be explained by the importance

of nucleic acid in the thymus. Pora & Toma (1960a) found that there is an increased elimination of ^{32}P in thymectomized animals, and they suggested that this may be because the inorganic phosphate that is no longer required by the thymus is found in the blood. Here some of it precipitates calcium. The acidosis and hypocalcaemia could explain the increase in excitability and decrease in chronaxie, conditions that are improved by treatment with thymus extracts.

The extracts from the thymus, fractions CIF, TP and TL, all had a similar effect on either calcium or phosphorus metabolism, with fraction TL having the most pronounced effect. Control muscle extract MP had no effect on phosphorus metabolism but did have an effect on calcium metabolism. Muscle extract ML behaved like fraction TL. Thus the effects of thymus extract TL are to be regarded as unspecific, and those of thymus extract TP as specific, with regard to effects on phosphorus metabolism, but not with regard to effects on calcium metabolism. Shibata (1953) and Potop, Felix & Ciocirdia (1959) have shown that extracts from another lymphatic organ, the spleen, do not affect phosphorus metabolism.

The results make evident the interrelation between calcium and phosphorus metabolism, a relationship considered also by other authors to be important in the nutrition and physiology of animals (Wasserman, 1960).

Correlations between muscle function and the activity of endocrine glands have been found by other workers studying, for example, the influence of oestradiol on the actomyosin content of uterus muscle (see Csapo, 1959) and on the content and

activity of mitochondrial and microsomal particles in uterus muscle (Wakid & Needham, 1961).

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