CLV. ENZYMIC SYNTHESIS OF COCARBOXYLASE IN ANIMAL TISSUES

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SOON after the discovery by Lohmann & Schuster [1937, 1] that cocarboxylase is the pyrophosphate of vitamin B₁, there were attempts by various workers to show that the vitamin can be enzymically phosphorylated to cocarboxylase by yeast and tissue preparations. Positive results were claimed with rat liver extracts by Euler & Vestin [1937], with acetone-dried preparations from pig's duodenal mucosa by Tauber [1937], and with avitaminous brain brei by Peters [1937, 1]; whereas Stern & Hofer [1937] failed to obtain synthesis using glycerol extracts from various rat tissues (liver, brain, intestine). Later Lohmann & Schuster [1937, 2] also reported some synthesis with minced pigeon's brain. In all these experiments tissue samples were incubated with vitamin B₁ in phosphate buffer, boiled and approximately assayed for cocarboxylase by measurement of the CO₂ evolution with alkaline-washed yeast and pyruvate.

The finding that free vitamin B₁, whilst unable to replace cocarboxylase, strongly stimulates its action in yeast [Ochoa, 1938], invalidated the conclusions drawn from those experiments where comparison had been made of the activities of samples incubated without and with vitamin B₁ respectively [Euler & Vestin, 1937; Peters, 1937, 1] as the higher activity of the latter could be due to the effect of the vitamin on the yeast system itself. Later experiments, to be reported in this paper, made Tauber's claim [1937] untenable that synthesis occurs with acetone preparations of intestinal mucosa. More recently Goodhart & Sinclair [1939] have shown that blood from avitaminous pigeons can synthesize cocarboxylase from vitamin B₁.

Ochoa & Peters [1938, 1] developed a method for the determination of cocarboxylase with yeast in the presence of an excess of vitamin B₁; this not only eliminated the effect of any vitamin present in the samples but also increased the sensitivity of yeast to cocarboxylase about ten times. Using this method they showed that 30 min. after injecting vitamin B₁ into avitaminous pigeons and rats there is a marked accumulation of cocarboxylase in the liver whereas little or no increase occurs in muscle, heart or brain. The *in vivo* synthesis in liver was confirmed (with the thiochrome method) by Westenbrink & Goudsmit [1938] who also found synthesis in the kidney.¹ Curiously enough the cocarboxylase in liver, after the injection, reaches values which are not much above the normal; hence injection of vitamin into normal pigeons causes only slight increases of cocarboxylase [Ochoa & Peters, unpublished results]. This is why avitaminous birds have been mainly used in the experiments described in this paper, although good syntheses were occasionally obtained with normal tissues.

The *in vitro* experiments [preliminary report: Ochoa & Peters, 1938, 2] have confirmed the above results in their main lines and added further information as

¹ This has been confirmed in experiments on rats in this laboratory [Ochoa & Rossiter, unpublished].

to the mechanism of the enzymic synthesis of cocarboxylase. Some of the results with brain have already been reported in a previous paper [Banga *et al.* 1939]. The synthetic power of brain is very small indeed, yet it accounts fully for the catatorulin effect of vitamin B₁.

EXPERIMENTAL

The majority of the experiments were carried out with slices, brei or "dispersions" of tissues from avitaminous pigeons. In some cases normal birds or birds which had been on a rice diet ("rice-fed") for various periods, but did not yet show symptoms, were also used. The tissue was suspended in Ringer phosphate, generally 2 ml. of medium to 0.5 g. tissue. "Dispersions" were prepared by thoroughly grinding the minced tissue in an ice-cold mortar with 4 vol. of ice-cold Ringer phosphate; the mixture was pressed through muslin and the resulting fine suspension used. Sometimes the mixture was centrifuged, the resulting solution (extract) and the solid residue (suspended in Ringer) being used separately or the residue was resuspended in the extract.

The samples plus additions were shaken, mostly at 38°, either in 25 ml. conical flasks, or in Warburg bottles when O₂ uptake was being measured. After incubation the samples were heated to 100° for 2–3 min., cooled, centrifuged and the cocarboxylase determined in the centrifugate as described by Ochoa & Peters [1938, 1]. Control samples were incubated without vitamin B₁, which was only added at the end (in the maximum amount used in the experiment) just before boiling.

No attempt was made to provide for buffering when working at reactions more alkaline than pH 8.0. The Ringer phosphate was merely brought to the desired pH with NaOH, as it seemed preferable to avoid the use of buffers other than phosphate. Pyrophosphate was found to inhibit the cocarboxylase synthesis.¹

Results are expressed in μ g. cocarboxylase per g. fresh tissue or its equivalent when extracts, acetone powder preparations etc. are used.

Synthesis of cocarboxylase under various conditions

Table I shows the results of experiments with pigeon tissue preparations. The following points should be noted: (1) Of the various tissues tried (liver, brain, breast muscle, intestine) liver is the only one which shows a good capacity to phosphorylate added vitamin B₁ to cocarboxylase. (2) Liver slices, brei or "dispersion" show a similar degree of activity; liver extracts are much less active (exps. 12 and 13), whereas suspensions of acetone-dried liver, prepared after Tauber [1938], are inactive (exps. 13 and 14). (3) The synthesis of cocarboxylase is much reduced in the absence of O₂ (exps. 2 and 8). (4) The cocarboxylase synthesis reaches a maximum (which is generally not much higher than the amounts present in the normal tissue) beyond which it does not go, no matter how much vitamin B₁ is added. Thus in exp. 2 the same results are obtained with 20 as with 40 μ g. vitamin per g. tissue and 200 μ g. vitamin B₁, in exp. 16 produced no higher synthesis than 50 μ g. Hence the percentage of vitamin converted into cocarboxylase is higher with small amounts of the former. In exp. 3 the synthesis is only slightly lower with 10 μ g. than with 20 μ g. vitamin per g. tissue but the conversion is 28% in the former and only 18% in the latter case. (5) Addition of various substrates with the vitamin did not increase the extent of the cocarboxylase synthesis.

¹ This may be due to its depressing action on respiration [cf. Greig & Munro, 1939].

Table I. *Synthesis of cocarboxylase from vitamin B₁ in various preparations of pigeon's tissues*

Exp. no.	Condition of animals	Enzyme	pH	Time of incubation and temp.		Gas	Additions (per g. tissue)	Cocarboxylase $\mu\text{g. per g. tissue}$	
								Found	Synthesis
1	Avit.	Liver slices	7.3	1 hr.	38°	O ₂	Control 50 $\mu\text{g.}$ vitamin B ₁	0.40 5.10	— 4.70
2	Avit.	Liver slices	7.3	"	"	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁ 20 $\mu\text{g.}$ vitamin B ₁	0.50 4.90 5.15	— 4.40 4.65
						N ₂	40 $\mu\text{g.}$ vitamin B ₁	1.40	0.90
3	Avit.	Liver slices	7.3	"	"	O ₂	Control 20 $\mu\text{g.}$ vitamin B ₁ 10 $\mu\text{g.}$ vitamin B ₁	0.90 6.40 5.20	— 5.50 4.30
4	Normal	Liver slices	7.3	"	"	O ₂	Control 60 $\mu\text{g.}$ vitamin B ₁	2.60 6.10	— 3.50
5	Avit.	Brain slices	7.3	"	"	O ₂	Control 95 $\mu\text{g.}$ vitamin B ₁	0.25 0.55	— 0.30
6	Avit.	Muscle brei	7.3	"	"	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁	0.85 1.47	— 0.62
7	Avit.	Intestine*	7.3	"	"	O ₂	Control 15 $\mu\text{g.}$ vitamin B ₁	0.12 0.28	— 0.16
8	Avit.	Liver slices	7.3	$\frac{1}{2}$ hr.	38°	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁ 40 $\mu\text{g.}$ vitamin B ₁ 20 mg. glucose	0.65 3.95 3.65	— 3.30 3.00
						N ₂	40 $\mu\text{g.}$ vitamin B ₁ 40 $\mu\text{g.}$ vitamin B ₁ 20 mg. glucose	1.76 1.89	1.11 1.24
9	Avit.	Liver brei	7.3	"	"	O ₂	Control 50 $\mu\text{g.}$ vitamin B ₁ 50 $\mu\text{g.}$ vitamin B ₁ 45 $\mu\text{g.}$ ATP† 50 $\mu\text{g.}$ vitamin B ₁ 600 $\mu\text{g.}$ Pgl.‡ 50 $\mu\text{g.}$ vitamin B ₁ 45 $\mu\text{g.}$ ATP 600 $\mu\text{g.}$ Pgl.‡	1.46 4.22 4.22 3.48 3.22	— 2.76 2.76 2.02 1.76
10	Avit.	Liver slices	8.5	1 hr.	38°	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁	0.52 5.50	— 4.98
		Liver brei	8.5	"	"	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁	0.68 14.60	— 13.92
11	Avit.	Liver brei	8.5	"	"	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁	0.50 7.80	— 7.30
12	Avit.	Liver extract	8.5	"	"	O ₂	Control 40 $\mu\text{g.}$ vitamin B ₁ 40 $\mu\text{g.}$ vitamin B ₁ 20 mg. glucose 40 $\mu\text{g.}$ vitamin B ₁ 90 $\mu\text{g.}$ ATP† 40 $\mu\text{g.}$ vitamin B ₁ 20 mg. glucose 90 $\mu\text{g.}$ ATP†	0.72 2.25 2.25 1.28 1.76	— 1.53 1.53 0.56 1.04

Table I (cont.)

Exp. no.	Condition of animals	Enzyme	pH	Time of incubation and temp.		Gas	Additions (per g. tissue)	Cocarboxylase $\mu\text{g. per g. tissue}$	
								Found	Synthesis
13	Avit.	Liver brei	8.5	1 hr.	38°	O ₂	Control	0.60	—
							40 $\mu\text{g.}$ vitamin B ₁	8.50	7.90
		Liver extract	8.5	„	„	O ₂	Control	0.60	—
							40 $\mu\text{g.}$ vitamin B ₁	2.10	1.50
		Liver acetone powder	8.5	„	„	O ₂	Control	0.60	—
							40 $\mu\text{g.}$ vitamin B ₁	0.70	0.10
14	Avit.	Liver acetone powder	8.5	„	„	O ₂	Control	0.40	—
							30 $\mu\text{g.}$ vitamin B ₁	0.40	0.00
15	Normal	Liver dispersion	8.5	„	„	O ₂	Control	3.40	—
							100 $\mu\text{g.}$ vitamin B ₁	13.40	10.00
16	Rice-fed	Liver brei	8.5	„	„	O ₂	Control	1.14	—
							50 $\mu\text{g.}$ vitamin B ₁	9.65	8.51
							100 $\mu\text{g.}$ vitamin B ₁	9.90	8.76
							200 $\mu\text{g.}$ vitamin B ₁	8.80	7.66
							200 $\mu\text{g.}$ vitamin B ₁	7.15	6.01
							20 mg. glucose		
							200 $\mu\text{g.}$ vitamin B ₁	6.36	5.22
							10 mg. NaHDP§		
							200 $\mu\text{g.}$ vitamin B ₁	6.88	5.74
							25 mg. Na succinate		

* Minced duodenum.

† Phosphoglyceric acid P.

† Easily hydrolysable P, adenosine triphosphate.

§ Na hexosediphosphate.

Fig. 1.

Fig. 2.

Fig. 1. O₂ uptake and synthesis of cocarboxylase from vitamin B₁ in liver from "rice-fed" pigeons. 250 mg. tissue in 1 ml. Ringer phosphate. 10 $\mu\text{g.}$ vitamin B₁ added at time 0. Curve I: O₂ uptake at 28° (liver brei); pH 8.5. Curve II: cocarboxylase at 28° (liver brei); pH 8.5. Curve III: cocarboxylase at 38° (liver slices); pH 7.3. Gas O₂.

Fig. 2. O₂ uptake and synthesis of cocarboxylase from vitamin B₁ in breast muscle and brain brei from "rice-fed" pigeons at 28°. 250 mg. tissue in 1 ml. Ringer phosphate pH 8.5. 10 $\mu\text{g.}$ vitamin B₁ added at time 0. Curve I: O₂ uptake (muscle). Curve II: cocarboxylase (muscle). Curve III: O₂ uptake (brain). Curve IV: cocarboxylase (brain). Gas O₂.

The formation of cocarboxylase in liver preparations reaches a maximum in 30–60 min. After remaining constant for a short time, the cocarboxylase later decreases. This is shown, for liver brei, in Fig. 1, both at 28 and 38°; the O₂ uptake was measured in the experiment at 28°. In Fig. 2 similar experiments with

brain and muscle are reproduced; here the cocarboxylase synthesis is much less and there is no drop from the maximum reached.

Experiments with liver slices at various *pH* are given in Table II. The synthetic reaction has a markedly alkaline *pH* optimum of about 8.5.

Table II. *Synthesis of cocarboxylase from vitamin B₁ in slices of liver from avitaminous pigeons at various pH*

40 μ g. vitamin B₁ per g. tissue added. Incubation in O₂, 40 min. at 38°.

Original <i>pH</i> of medium	Cocarboxylase synthesized (μ g. per g. tissue)		
	Exp. 1	Exp. 2	Exp. 3
6.2	3.32	—	3.47
6.8	4.09	—	—
7.3	5.08	—	—
8.0	5.42	8.31	5.07
8.5	—	12.51	5.51
9.0	—	10.51	—
9.5	—	11.41	5.07
10.0	—	5.21	4.24

Preparations from intestinal mucosa

Tauber [1937; 1938] claims to have obtained synthesis of cocarboxylase with acetone-dried preparations of duodenal mucosa from the pig. Exp. 7 of Table I shows that with pigeon's intestine hardly any synthesis is obtained. It has already been noted that acetone preparations of liver made by Tauber's method are inactive (Table I, exps. 13 and 14). In view of this, some experiments were done with pig's duodenal mucosa using the fresh minced mucosa, extract in Ringer phosphate and a suspension of the acetone-treated material after Tauber. In all cases the intestine from freshly slaughtered pigs was immediately brought into the laboratory on ice and the preparation of the mucosa was started at once. On incubation of the various preparations with vitamin B₁ for 1 hr. at 38° in O₂, at the optimum *pH* for liver (8.5), no synthesis of cocarboxylase was found (Table III). In his experiments Tauber [1937; 1938] used very long incubation periods of 20 hr. and more and found the optimum reaction to be

Table III. *Absence of cocarboxylase synthesis with preparations from pig's duodenal mucosa*

Incubation in O₂ at 38°, 1 hr. Medium: Ringer phosphate *pH* 8.5. 200 mg. acetone powder (= 2 g. fresh mucosa) in 4 ml. Ringer.

Preparation	Additions per g. tissue	Cocarboxylase (μ g. per g. tissue)	
		Found	Change
Brei	Control	0.52	—
	40 μ g. vitamin B ₁	0.50	- 0.02
Extract	Control	0.31	—
	40 μ g. vitamin B ₁	0.31	0.00
Acetone powder	Control	0.26	—
	40 μ g. vitamin B ₁	0.28	+ 0.02

approximately *pH* 6.8. He worked, as far as possible, under sterile conditions but it is impossible to sterilize the enzyme suspension satisfactorily. When Tauber's experiment was repeated a large synthesis of cocarboxylase was found to have taken place after 20 hr. incubation, but the samples taken at various intervals, up to 8 hr., showed no synthesis whatever; on the contrary, there was a slight and gradually increasing breakdown of the cocarboxylase originally

present (Table IV). This result indicates that the synthesis after 20 hr. must have been due to growing bacteria. If the mucosa preparation had contained an active enzyme, the synthesis of cocarboxylase would be expected to have started earlier and increase gradually.

Table IV. *Cocarboxylase in acetone preparations of pig's duodenal mucosa incubated for various periods with vitamin B₁*

Each sample contained 200 mg. acetone powder in 4 ml. 0.1 M phosphate buffer pH 6.8 with 0.4 mg. vitamin B₁ HCl. Incubation in air at 33°.

Incubation time hr.	Cocarboxylase (μg. per sample)	
	Found	Change
0	1.4	—
1½	0.7	- 0.7
3	0.6	- 0.8
5	0.5	- 0.9
8	0.4	- 1.0
20	26.0	+ 24.6

It is now well known that bacteria can synthesize cocarboxylase from vitamin B₁. This has been shown by the recent experiments of Silverman & Werkman [1939] and Barron & Lyman [1939]. Earlier experiments by Hills [1938] and Silverman & Werkman [1938] already indicated this possibility.

Relation of cocarboxylase synthesis to respiration

The experiments of Table I show that the synthesis of cocarboxylase is very small in liver extracts, and also in more active liver preparations if O₂ is absent. The low activity of the extracts is due to the fact that their O₂ uptake is very small, but the synthesizing enzyme is soluble and is present in them. The experiment of Table V shows this very clearly. The samples contained liver extract, the remaining solid residue suspended in Ringer, and residue suspended either in extract or in boiled extract. In the cocarboxylase synthesis experiment the liver used was from avitaminous pigeons; the O₂ uptake was measured in similar preparations of liver from normal pigeons. It will be seen that the

Table V. *Synthesis of cocarboxylase from vitamin B₁ in liver extracts, liver residue and in both combined (liver from avitaminous pigeons) and O₂ uptake of the same fractions from normal pigeon's liver*

1 hr. at 38°, pH of medium 8.5. 40 μg. vitamin B₁, HCl per g. tissue added to each sample.

No.	Sample	Cocarboxylase synthesized	μl. O ₂ uptake per g. tissue
		μg. per g. tissue	
1	Residue in Ringer	0.25	8
2	Extract (4 ml.)	1.72	114
3	Residue in extract	5.20	1630
4	Residue in boiled extract	0.80	1825

combination of residue and extract gives both a good cocarboxylase synthesis and a high respiration. The residue alone or the extract alone give neither. The residue does not respire owing to lack of soluble substrates and coenzymes which can be supplied with boiled extract; the respiration is then restored but not the power to synthesize cocarboxylase, proving that the synthesizing enzyme was present mainly in the extract and was destroyed by boiling.¹

¹ This does not exclude the possibility that some other necessary factor might be present in the residue.

The amount of preformed cocarboxylase in normal pigeon's liver brei, if this is incubated in Ringer phosphate pH 8.5 at 28°, over a period of 4 hr., remains absolutely constant in O₂ but decreases steadily on incubation in N₂. Thus in one case the brei contained 5.4 µg. cocarboxylase per g. at the start of the experiment¹ and 5.7 µg. at the end of 4 hr. in O₂, but only 2.9 µg. per g. at the end of the same period in N₂. The decrease was 47 %. The O₂ uptake in this experiment increased linearly with time and reached 8000 µl. O₂ per g. tissue at the end of the 4 hr. period. The breakdown of cocarboxylase when O₂ is withdrawn might be partially responsible for the damaging effect of anaerobiosis on the pyruvate oxidation system of brain observed by Peters [1937, 2; cf. especially p. 11].

Effect of poisons on the synthesis of cocarboxylase

The effects of iodoacetic acid and NaF on the synthesis of cocarboxylase in liver slices are shown in Table VI. The synthesis is markedly inhibited by iodoacetate (0.0009 M) but only slightly affected by NaF (0.04 M).

Table VI. *Effect of poisons on the synthesis of cocarboxylase from vitamin B₁ in liver slices from avitaminous pigeons*

pH of medium 7.3. Incubation in O₂ at 38°, exp. 1, 30 min.; exp. 2, 50 min.

Exp. no.	Additions per g. tissue	Cocarboxylase µg. per g. tissue		Inhibition of synthesis %
		Found	Synthesized	
1	Control	1.00	—	—
	40 µg. vitamin B ₁	6.42	5.42	—
	Vitamin + NaF (0.04 M)	5.30	4.30	21
	Vitamin + IAA (0.005 M)	1.11	0.11	98
2	Control	0.61	—	—
	200 µg. vitamin B ₁	3.93	3.32	—
	Vitamin + NaF (0.04 M)	3.33	2.72	18
	Vitamin + IAA (0.0009 M)	1.63	1.02	70

Breakdown of cocarboxylase in tissues

The tissues contain a soluble enzyme (probably a phosphatase) which destroys cocarboxylase. In tissue extracts where, owing to lack of respiration, no resynthesis of cocarboxylase can take place, breakdown of preformed or

Table VII. *Cocarboxylase breakdown in tissue preparations*

Incubation at 38°. Cocarboxylase added in exps. 1, 2 and 3.

Exp. no.	Animal	Enzyme	pH	Incubation time hr.	Gas	Cocarboxylase µg. per g. tissue		% breakdown
						Before	After	
1	"Rice-fed" pigeon	Liver extract	8.5	2	O ₂	5.7	1.5	73
2	"Rice-fed" pigeon	Liver extract	7.5	1	O ₂	33.5	11.0	67
3	"Rice-fed" pigeon	Liver extract*	7.5	1	O ₂	26.4	8.5	68
4	Normal pigeon	Brain disp.†	8.5	4	N ₂	2.61	1.84	29.5
		Muscle disp.†	8.5	4	N ₂	2.63	2.23	15.1
		Liver disp.†	8.5	4	N ₂	3.84	1.22	68.0
		Kidney cortex disp.†	8.5	4	N ₂	4.22	0.92	76.3
5	Normal rabbit							

* In exp. 3 an aqueous liver extract was precipitated with 10 vol. acetone. The dried acetone powder was dissolved in phosphate buffer and this extract used, after centrifuging off from insoluble material.

† "Disp." = dispersion.

¹ In order to secure a high liver cocarboxylase, the bird used in this experiment was injected with 3 mg. vitamin B₁ 30 min. before killing it.

added cocarboxylase occurs even in the presence of O_2 ; in minced tissues it takes place when O_2 is withdrawn. The powers of the various tissues to destroy cocarboxylase are different; liver and kidney are much more active in this respect than are skeletal muscle or brain (Table VII), thus breakdown is highest in those tissues which show the highest activity to synthesize cocarboxylase from vitamin B_1 .

DISCUSSION

The results presented in this paper would be best explained by assuming that one and the same enzyme catalyses the reaction

When energy is available (i.e. from respiration) the equilibrium is shifted towards the left. There must however be another, as yet unknown, factor which limits the extent of the synthetic reaction; this makes a further analysis of the reaction impossible and therefore the above view can only be advanced as a provisional hypothesis.

It is possible that esterification of the vitamin with inorganic phosphate is coupled with oxidation of triosephosphate,¹ the inhibition of the synthesis by iodoacetic acid is in favour of this view; but whether this would involve adenylic acid in an intermediate reaction it is impossible to decide. Failure to obtain increased synthesis with liver preparations (which are not synthesizing maximally; cf. Table I, expts. 9 and 12) on addition of adenosine triphosphate, phosphoglyceric acid, or both together, is evidence against a direct transfer of phosphate from either compound.

In yeast Lipschitz *et al.* [1938] think that phosphorylation of the vitamin may be coupled with triosephosphate oxidation. Through the kindness of Dr Lipschitz I have been able to test the ability of their yeast to synthesize cocarboxylase from vitamin B_1 after alkaline washing (unpublished experiments), and found that synthesis does occur but only to a small extent, not more than with liver preparations. In the presence of pyruvate, synthesis is dependent upon the presence of cozymase but the presence of hexosediphosphate is not essential. These points obviously require further investigation.

The physiological significance of the higher synthetic activity of liver (and kidney) as compared with other tissues, and the possible conveyance of vitamin B_1 as such in the blood, have been discussed in a previous paper [Banga *et al.* 1939; cf. Goodhart & Sinclair, 1939].

SUMMARY

1. Liver slices, brei or "dispersions" from avitaminous pigeons convert added vitamin B_1 into cocarboxylase to an extent which does not greatly surpass the normal cocarboxylase content of the tissue. Optimally, with small amounts of vitamin, 30 % of this may be converted into cocarboxylase in 30–60 min. at 38°.

2. Brain and muscle preparations are much less active; preparations from duodenal mucosa (pig) showed no activity under various experimental conditions.

3. With liver, synthesis is dependent on an active respiration and is highest at alkaline reactions (optimum about pH 8.5). The synthesis is inhibited by iodoacetic acid but not much affected by NaF.

4. An essential part of the enzyme catalysing the synthesis is soluble.

¹ The fact that synthesis with liver is much reduced in N_2 may be due to the low glycolytic activity of liver preparations.

5. Soluble enzymes which, when no respiration is possible, destroy cocarboxylase, are present in liver, kidney, muscle and brain; their activity is highest in those tissues (liver and kidney) which also show the highest synthetic capacity.

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