CCXLVII. FLAVIN-ADENINE-DINUCLEOTIDE IN RAT TISSUES[1]

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It now seems clear that flavin-adenine-dinucleotide acts as the coenzyme or "prosthetic group" of at least five enzymes of biological interest: (1) *d*-amino acid oxidase [Warburg & Christian, 1938], (2) the flavoprotein from milk of Corran & Green [1938] which is at all events similar to the xanthine oxidase of Ball [1939], (3) the yeast flavoprotein of Haas [1938], (4) diaphorase of Euler & Hellström [1938], or coenzyme factor of Dewan & Green [1938] (cf. also Straub [1939]; Straub *et al.* [1939]), and (5) yeast fumarate hydrogenase [Fischer *et al.* 1939]. In view of the great importance of this nucleotide it seemed of interest to determine the amount present in boiled extracts of various rat tissues under different experimental conditions.

Vivanco [1935, 1, 2] has described a lowering of the lactoflavin content of rat tissues (especially heart and liver) following the feeding of a flavin-free diet. Similar results have been reported for liver [Kuhn *et al.* 1935], and for liver and possibly heart [Groen & Schuzl, 1938]. We shall show that there is a similar decrease of the flavin-adenine-dinucleotide in heart and liver tissue.

Experimental methods

The flavin-adenine-dinucleotide was determined by the method of Warburg & Christian [1938], the O_2 utilized in the oxidative deamination of alanine being measured manometrically. The nucleotide-free enzyme was prepared from a kidney acetone powder, and partially purified as described by Negelein & Brömel [1939]. The separation of the flavin-adenine-dinucleotide was done according to Warburg & Christian's [1938] directions. In the final stage the enzyme from 35 g. acetone powder was dissolved in 7 ml. *M*/15 pyrophosphate buffer *p*H 8·3 and diluted with ice-cold water to 90 ml.

The acetone powder was moderately stable, and also the entire enzyme solution if kept at 0°. In absence of coenzyme solutions were only stable for one week.

To each manometric bottle were added to the main chamber: (1) 1 ml. pyrophosphate buffer *p*H 8·3, (2) 0·4 ml. enzyme solution, (3) 0·5 ml. solution to be estimated, or standard flavin-adenine-dinucleotide. The side bulb contained 0·2 ml. 4·5 % solution of *dl*-alanine. KOH-filter papers were placed in the central well. Air filled the gas space and the temperature was 38°. The substrate was tipped in when the bottles were placed in the bath and the O_2 uptake was

[1] A preliminary report of some of these experiments has been published in *Nature, Lond.*, 1939, **144**, 787.

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measured for a 30 min. period commencing exactly 15 min. later. During this period the rate of O_2 uptake remained constant.

The relation between O_2 uptake in 30 min. and added flavin-adenine nucleotide in our experiments is shown in Fig. 1. For amounts of nucleotide less than 1 μg. the relation between O_2 uptake and nucleotide added is almost linear.

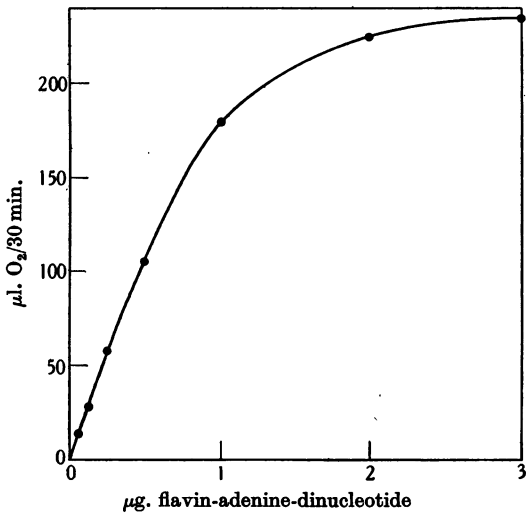

Fig. 1. The relation between O_2 uptake and flavin-adenine-dinucleotide added.

Solutions to be determined were always diluted so that the nucleotide content fell within this range. For each series of determinations at least two bottles containing known amounts of a standardized solution were used to correct for daily fluctuation in the enzyme activity.

Preparations

Flavin-adenine-dinucleotide. A sample of the pure nucleotide kindly sent by Prof. O. Warburg to Prof. R. A. Peters.

Vitamin B_1 hydrochloride. Synthetic specimens kindly supplied by Messrs Hoffmann La Roche and Messrs Bayer.

Lactoflavin and alanine. These were commercial preparations. The lactoflavin was flavin-adenine-dinucleotide-free.

1. *The flavin-adenine-dinucleotide content of boiled extracts of tissues from normal rats*

The rats, males from the usual laboratory stock, were fed on a synthetic diet supplemented with whole yeast and cod liver oil. They were killed by decapitation, bled, the tissues weighed in a weighing bottle, finely minced with scissors and ground with 5 volumes distilled water in a small porcelain mortar. The tissue suspension was then plunged into a water bath at 80° for 15 min., cooled, centrifuged and suitably diluted. Table I gives the average values found. The individual values are given in Table VIII of the appendix.

Table I. *Mean flavin-adenine-dinucleotide content (μg./g. fresh tissue) in tissues of normal and adrenalectomized rats*

Condition of animal	No. of animals	Tissue	Mean	2 × S.E. mean
Normal	6	Brain	10·0	± 0·8
	6	Heart	64·5	±20·8
	6	Kidney	61·0	±11·2
	8	Liver	77·3	±11·4
Adrenalectomized	6	Brain	10·3	± 0·6
	5	Heart	69·4	±15·8
	6	Kidney	58·7	± 7·4
	6	Liver	75·5	±10·2

2. *Flavin-adenine-dinucleotide content of boiled extracts of tissues of rats on a diet deficient in flavin*

Two groups of male rats (average weight 35 g.) were fed on the following diet:

Rice starch	70
Casein (alcohol-extracted)	20
Salt mixture	5
Agar-agar	2
Cod liver oil	3

The diet was supplemented by the following per rat per day:

10 μg. crystalline vitamin B_1 hydrochloride;
0·2 ml. 50% acid-alcohol yeast concentrate;
0·2 ml. liver filtrate factor.

The yeast concentrate was prepared as described by Kinnersley *et al.* [1933], and the liver filtrate was previously treated with fuller's earth and franconite. Both were prepared by Mr L. A. Stocken, to whom our sincere thanks are due. This diet is reasonably free from flavin, especially if care is taken with the extraction of the casein.

Group A (Fig. 2) fed on this diet increased in weight but little, while group B, which received in addition a daily supplement of 50 μg. lactoflavin per animal, showed a normal growth rate. After 30 days the animals were killed and the flavin-adenine-dinucleotide determined as before (Table II). The individual

Table II. *Mean flavin-adenine-dinucleotide content (μg./g. fresh tissue) of rat tissue*

Group	Diet	No. of animals	Mean wt. change (g. ±2 × S.E. mean)	Tissue	Mean ±2 × S.E. mean
A	Flavin-deficient	6	14·9 ± 4·0	Brain	10·3 ± 0·4
				Heart*	38·2 ± 3·6
				Kidney	72·5 ±10·8
				Liver*	41·4 ± 6·6
B	Flavin-deficient plus 50 μg. lactoflavin per day	6	79·8 ±14·0	Brain	12·8 ± 2·2
				Heart	92·7 ±17·8
				Kidney	78·4 ±15·4
				Liver	87·0 ±22·2

* Heart and liver figures are mean for 9 animals.

results are given in the appendix (Tables IX and X). It is seen that the mean nucleotide content of the tissues of deficient rats is lower than that of those

receiving adequate amounts of flavin. This is significantly so in the case of heart and liver tissue. Application of Fisher's "t" test to the difference of means of the heart and liver figures gives $t=7{\cdot}28$ and $4{\cdot}65$ respectively. For $P=0{\cdot}01$, $t=3{\cdot}01$. The figures are thus clearly significant. The results are represented graphically in Fig. 3.

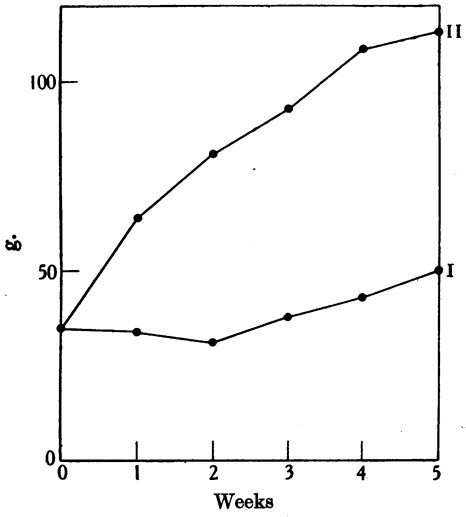

Fig. 2.

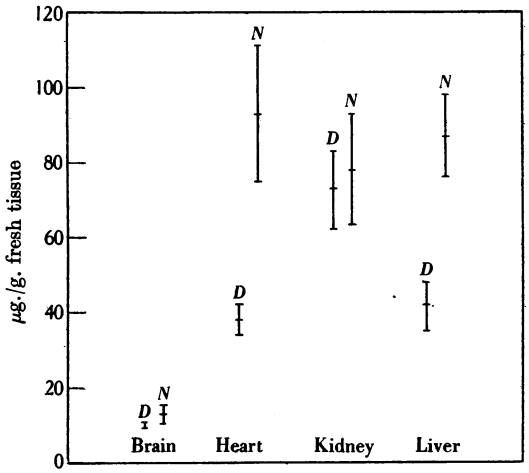

Fig. 3.

Fig. 2. Changes in weight of rats on flavin-deficient diet. Each curve = average for 6 rats. Curve I: diet only. Curve II: diet plus 50 μg. lactoflavin per day.

Fig. 3. Flavin-adenine-dinucleotide (μg./g. fresh tissue) in rat tissue. *D*, flavin-deficient diet. *N*, normal (i.e. flavin-deficient diet plus 50 μg. lactoflavin per day). Ordinate; mean $\pm 2 \times$ S.E. mean.

Some animals on the flavin-deficient diet developed a dermatitis, especially around the eyes, and others (results not included in the tables) died before the conclusion of the experiment. The boiled extracts from the organs of these animals still contained appreciable amounts of the nucleotide.

3. *The* in vivo *synthesis of flavin-adenine-dinucleotide from lactoflavin*

The flavin-adenine-dinucleotide content of liver and heart tissues of rats on a flavin deficient diet can be increased (Table III) by injection of lactoflavin. After injection of 1 mg. lactoflavin on each of three occasions, 24, 4 and ½ hr. before killing, the nucleotide content of these organs approximates to normal values. This constitutes an *in vivo* synthesis of the nucleotide from lactoflavin.

In animals killed ½ hr. after the first injection, however, a rise was observed in the nucleotide content of the liver, but not in the heart. This seems to indicate that the liver is able to synthesize the flavin-adenine-dinucleotide more readily than the heart. It will be seen from the table that the simultaneous injection of adenylic acid had practically no effect on the nucleotide content of the tissues. In the kidney and brain tissues of deficient rats, in which the flavin-adenine-dinucleotide content is practically normal, no further increase was observed after flavin injection.

Table III. *Flavin-adenine-dinucleotide content (μg./g. fresh tissue) of tissue from rats on flavin-deficient diet, before and after injection of lactoflavin and adenylic acid*

Treatment of flavin-deficient animals	Liver	Heart
Mean ±2 × S.E. 9 uninjected animals	41·4 ±6·6	38·2 ±3·6
Injected 1 mg. lactoflavin ½ hr. before death	56	40·5
	59	42
Injected 1 mg. lactoflavin + 1 mg. adenylic acid ½ hr. before death	58	38
	62	—
Injected 1 mg. lactoflavin on each of three occasions, 24, 4 and ½ hr. before death	67	58
	68	62
	57	62
Injected 1 mg. lactoflavin + 1 mg. adenylic acid on each of three occasions, 24, 4 and ½ hr. before death	69	53
	65	59

4. *Flavin-adenine-dinucleotide in boiled extracts from tissues of adrenalectomized rats*

Verzár *et al.* [1937] reported a diminution in bound (non-dialysable) and an increase in free (dialysable) flavin in the liver of rats following adrenalectomy. Later, Laszt & Verzár [1938] obtained similar results for liver, kidney and heart tissue of the cat. Since the flavin-adenine-dinucleotide probably represents the greater part of the non-dialysable flavin in liver tissue it seemed of interest to determine the amount of the nucleotide present in the tissues of rats suffering from adrenal cortical insufficiency.

The adrenals were removed from male rats by the technique described by Firor & Grollman [1932], under ether anaesthesia. The rats were of the same age-

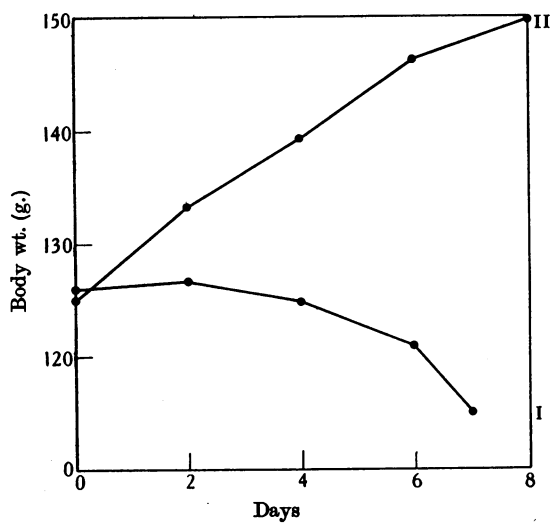

Fig. 4. Changes in weight of rats following adrenalectomy. Each curve = average for 6 rats. Curve I: adrenalectomized at day 0. Curve II: normal controls.

group and received the same diet as those described in section 1. The animals were killed when they were "in extremis" showing the typical signs—extreme weakness, loss of appetite, low body temperature, and loss of body weight (Fig. 4)—

of adrenal cortical insufficiency. Table I shows that there was no significant decrease in the flavin-adenine-dinucleotide content of the tissues of such animals. Individual values are given in the appendix (Table XI).

5. *Flavin-adenine-dinucleotide in blood*

The nucleotide content of three samples of ox blood is given in Table IV. It is seen that there is no measurable activity in unheated blood. Heating for 3 min. at 100° renders the blood active. This observation is similar to that of Goodhart & Sinclair [1939] for the cocarboxylase activity of blood, and can

Table IV. *Flavin-adenine-dinucleotide in ox blood (μg./100 ml.)*

Whole blood (unheated)	Whole blood (heated at 100° for 3 min.)	Plasma (unheated)	Plasma (heated at 100° for 3 min.)
0	55	0	22·5
0	55	0	25·5
2	49	0	17

possibly be interpreted in the same fashion, viz. the liberation of an active substance from a protein complex. They found, however, that the cocarboxylase activity was limited to the cells. Our observations show that the blood plasma contains some flavin-adenine-dinucleotide. The temperature, time and *p*H of heating were all found to be important. The usual practice was to heat the blood at 100° for 3 min. at *p*H 7. The fact that in the presence of alanine no O_2 is absorbed by unheated blood or plasma shows that it has no amino-acid oxidase activity.

Table V. *Flavin-adenine-dinucleotide in rat blood (μg./100 ml.)*

Normal	Flavin deficient	Flavin-deficient after injections of 1 mg. lactoflavin on each of three occasions, 24, 4 and ½ hr. before death
64·5	52·5	61
56	48	61·5
64·5	52·5	—
63·5	—	—
65	—	—

Table V gives the flavin-adenine-dinucleotide values for the blood of 5 normal, 3 deficient, and 2 deficient rats which had been injected with 1 mg. lactoflavin on each of three occasions, 24, 4 and ½ hr. before killing. The figures suggest a diminution in nucleotide content of the blood in flavin deficiency, and that this deficiency is rapidly (within 24 hr.) made good on injecting flavin.

6. *The enzymic breakdown of flavin-adenine-dinucleotide*

Incubation of tissue (usually liver) from normal and flavin-deficient animals in the presence of flavin and adenylic acid never showed a synthesis of the nucleotide; in fact, there was usually a breakdown of the nucleotide already present in the tissue. This breakdown is enzymic and Table VI shows it to be dependent on the *p*H of the medium.

Table VI. *Flavin-adenine-dinucleotide in liver slices (μg./g. fresh tissue) after incubation for 3 hr. at various* p*H. Temp. 38°*

No incubation	*p*H 6	*p*H 7·3	*p*H 8·4
53	40·5	32·5	20
45	41	19	10
46	38	30	9·5

Table VII shows the relative rates of nucleotide breakdown with "dispersion" preparations from brain and liver tissue after aerobic incubation at 38° and *p*H 8·3. It is seen that in the brain there is no significant breakdown after 3 hr. incubation, but that in the liver the breakdown is much more rapid. This is similar to the observations of Ochoa [1939] on breakdown of cocarboxylase.

Table VII. *Relative breakdown of flavin-adenine-dinucleotide (μg./g. fresh tissue) in suspensions of brain and liver tissue.* p*H 8·4. Temp. 38°*

Tissue	No incubation	½ hr.	1 hr.	2 hr.	3 hr.
Brain	12·5	12	12	12	12
	11·5	11	11	11	10·5
Liver	59	57	51	49	42
	72	67	61	56	47

Discussion

The figures given for the flavin-adenine-dinucleotide content of normal rat tissues are of the same order as those of Warburg & Christian [1938]. If the mol. wt. of the nucleotide is taken as 785 and that of lactoflavin as 376, the results, molecule for molecule, are also of the same order as those reported for the lactoflavin content of rat tissues by Vivanco [1935, 1, 2] and a little higher than those of Kuhn *et al.* [1935], Verzár *et al.* [1937], Gourévitch [1937] and Groen & Schuzl [1938]. This would indicate that probably the major portion, and possibly all, of the flavin so measured was in the form of the nucleotide.

Verzár *et al.* [1937] explain their observations on adrenalectomized animals by suggesting that in adrenal cortical insufficiency there is a diminution in the proportion of the total flavin which is present in the phosphorylated form. The fact that the flavin-adenine-dinucleotide (a phosphorylated form of flavin) seems to account for most of the flavin present, and is not diminished after adrenalectomy, is difficult to correlate with the interpretation of Verzár *et al.* although it is consistent with our observations on cocarboxylase [Ochoa & Rossiter, 1939].

The decrease of the flavin-adenine-dinucleotide in the liver following a flavin-deficient diet is parallel to the decrease of cocarboxylase following a vitamin B_1-deficient diet [Ochoa & Peters, 1938], and to the decrease in cozymase [Axelrod & Elvehjem, 1939] and of V-factor [Kohn *et al.* 1939] after feeding a diet deficient in nicotinic acid. With cocarboxylase there is a general decrease in all tissues, but in the case of V-factor the decrease was observed in liver and skeletal muscle only. Similarly, the decrease in flavin-adenine-dinucleotide was observed only in liver and heart (skeletal muscle was not examined). This would be consistent with the view that the liver acts as a store, at the expense of which the levels in the other tissues are maintained. It is of interest to note that Groen & Schuzl [1938] obtained a diminution in O_2 uptake with liver tissue from rats on a flavin-deficient diet, and that Axelrod *et al.* [1939] have recently reported a similar decrease in O_2 uptake of liver and kidney tissue with alanine as substrate, suggesting a diminution of the flavin-adenine-dinucleotide in these tissues. The decrease in the nucleotide content of blood, paralleled by the decrease in cocarboxylase in pigeon's blood [Goodhart & Sinclair, 1939], and of the V-factor in the blood of pellagrins [Vilter *et al.* 1939], is also of interest. Kohn *et al.* [1939 for references], however, found no such decrease in the V-factor in the blood of pellagrins or of dogs suffering from acute blacktongue.

The results reported here afford a further example of a deficiency of a vitamin of the B-complex affecting important biological oxidation systems.

Summary

1. The flavin-adenine-dinucleotide content of boiled extracts of brain, heart, kidney and liver tissue of normal rats has been determined.

2. There is a decrease in the flavin-adenine-dinucleotide content of boiled extracts of liver and heart tissue from rats fed on a flavin-deficient diet. No significant difference could be detected in extracts of brain and kidney tissue.

3. Injection of 1 mg. lactoflavin on each of three occasions, 24, 4 and $\frac{1}{2}$ hr. before death restores the flavin-adenine-dinucleotide content of liver and kidney tissue of flavin-deficient rats to normal levels. This constitutes an *in vivo* synthesis of the nucleotide from lactoflavin. The restoration is more rapid in the liver than in the heart.

4. There is no significant difference between the flavin-adenine-dinucleotide contents of boiled extracts of tissues from normal and adrenalectomized rats.

5. There is no flavin-adenine-dinucleotide activity in unheated blood (ox, rat), but this activity is present after heating the blood at 100° for 3 min. at *p*H 7. The flavin-adenine-dinucleotide in the blood of flavin-deficient rats is lower than that of normal animals, but is restored by injection of 1 mg. lactoflavin on each of three occasions, 24, 4, $\frac{1}{2}$ hr. before death.

6. The enzymic breakdown of the flavin-adenine-dinucleotide in the tissues was found to be dependent upon *p*H. Breakdown is more rapid with liver than with brain tissue.

We are greatly indebted to Prof. R. A. Peters for his continued interest and encouragement throughout this work.

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APPENDIX

Table VIII. *Flavin-adenine-dinucleotide in tissues of normal rats (μg./g. fresh tissue)*

Brain	Heart	Kidney	Liver
9·5	69	55	78
8·5	78	56	78
10	45	55	51·5
9	106	89	97
11	46	59	72
10·5	43	51·5	58
—	—	—	94
—	—	—	89

Table IX. *Flavin-adenine-dinucleotide in tissues of rats on flavin-deficient diet (μg./g. fresh tissue)*

Brain	Heart	Kidney	Liver
11	31	61	28
9·5	30·5	70	36·5
11	36·5	69	36
10	40	86	40·5
10	47	91	64
9·5	40·5	58	37·5
—	43	—	42
—	35	—	40
—	39	—	47

Table X. *Flavin-adenine-dinucleotide in tissues of rats on flavin-deficient diet plus 50 μg. lactoflavin per day (μg./g. fresh tissue)*

Brain	Heart	Kidney	Liver
10	52	51·5	56
8·5	89	84	62
15	115	83	107
14	94	92	80
12·5	100	—	89
15·5	106	81	128

Table XI. *Flavin-adenine-dinucleotide in tissues of adrenalectomized rats (μg./g. fresh tissue)*

Brain	Heart	Kidney	Liver
10	64	55	64
9	97	75	97
11	66	58	73
10·5	—	56	66
11	62	61	69
9·5	58	47	84

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