CLXXVI. THE V-FACTOR CONTENT AND OXYGEN CONSUMPTION OF TISSUES OF THE NORMAL AND BLACKTONGUE DOG

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It is now well established that nicotinic acid acts as a vitamin for a number of species of mammals and bacteria, and that its amide is combined in the molecule of two coenzymes which play a major part in intracellular oxidation. The question arises whether there is any connexion between these two functions. In this paper we describe experiments designed to provide information upon this point. We have determined quantitatively the V-factor which we use as a measure of the coenzyme content of the tissues in normal dogs and dogs suffering from acute deficiency of nicotinic acid ("blacktongue"), and have also measured *in vitro* the O₂ consumption of some of these tissues in the presence of suitable substrates. Our results show that the synthesis of V-factor can be restricted by greatly decreasing the intake of nicotinic acid but do not suggest that blacktongue can be explained in the simple terms of a generalized coenzyme deficiency.

The vitamin activity of nicotinic acid in curing specific deficiency diseases was first reported for the dog by Elvehjem *et al.* [1937] and extended to man by Fouts *et al.* [1937], Harris [1937] and Smith *et al.* [1937], to swine by Chick *et al.* [1938], and to the monkey by Harris [1937, 1938]. Although no specific deficiency disease has been shown to result from deprivation of dietary nicotinic acid in other species, there is evidence that it is essential for the guinea-pig [Harris, 1939] and the pigeon [Funk & Funk, 1937; Harris, 1939]. The earliest reports on micro-organisms showed that nicotinic acid is essential for the growth of *Staphylococcus aureus* [Knight, 1937] and the diphtheria bacillus [Mueller, 1937], and similar observations have since been made for the dysentery bacillus [Koser *et al.* 1938] and for *Proteus* [Fildes, 1938].

Nicotinamide was shown by Warburg to be combined in the molecule of codehydrogenases. [For brevity coenzyme I or cozymase will be designated DPN (diphosphopyridinenucleotide) and coenzyme II as TPN (triphosphopyridinenucleotide).] The precise quantitative evaluation of the parts played by DPN and TPN in cellular metabolism has not been made, but the basic importance of these substances seems to be beyond question. Their structure and definition have been reviewed by Euler [1936] and Warburg [1938], and the number, type and relationships of the systems in which they function have been discussed recently by Fischer [1939], Dixon [1939] and Martius [1939]. For our present purpose it should be recalled that the oxidation of glucose may involve either DPN or TPN, probably the latter more often. DPN is required for glycolysis resulting in lactic acid formation by muscle and probably all other tissues [cf. Meyerhof & Ohlmeyer, 1937], and for the oxidation of lactate by

kidney, heart, liver, brain, lung, diaphragm and skeletal muscle [Green & Brosteaux, 1936; Quastel & Wheatley, 1938].

A preliminary report on V-factor level in the blood of the dog has already been published by us [Kohn & Dann, 1939].

Experimental methods

Care and feeding of the dogs. The dogs were housed in metal cages having removable tray bottoms which were liberally filled with pine shavings. The trays were removed, cleaned and filled with fresh shavings every alternate day. Some of the dogs were removed for certain periods to metabolism cages exactly similar except for the replacement of the bottom tray by an openwork metal screen which allowed collection of the urine. The food and water troughs were attached to the sides of the cages in order to prevent fouling by urine or faeces. Adult dogs of both sexes and mixed breeds were used, weighing about 10 kg. each.

Two diets were used during the course of this study; these are referred to as the stock diet and the deficient diet. The stock diet consisted of horse meat, bread and cowpeas, cooked together. The horse meat included all the muscle meats of the carcass together with the bones and all other organs, except the intestines and the liver. The deficient regime was a modified Goldberger diet with certain vitamin supplements. The diet was prepared as follows: yellow cornmeal 400 parts, ground cowpeas 50 parts, Underhill & Mendel's [1928] salt mixture 12 parts and water 2000 parts were cooked for 2 hr. in an open boiler; then alcohol-extracted casein 60 parts, sucrose 32 parts and cottonseed oil 30 parts by weight were stirred into the mixture. Each dog was given 1 kg. of this food daily and its consumption was recorded. The diet was supplemented by weekly treatment with capsules of shark liver oil (40,000 International Units of vitamin A) and 1 mg. aneurin chloride orally.

Some of the dogs used in the blood study were maintained on the deficient diet until they developed blacktongue and were then cured with a small dose of nicotinamide; blood samples were taken at intervals before, during and after the attack. Others received either the deficient diet or the stock diet and were given in addition daily subcutaneous injections of nicotinic acid, either 2 mg. or 20 mg. per kg. body weight.

The dogs killed for tissue analysis fall into three groups. Those with blacktongue form Group BT. A dog was considered to be suffering from acute blacktongue when at least three of the following signs occurred together: (1) Marked reddening or necrosis of the membranes of mouth or throat. (2) Complete or almost complete loss of appetite. (3) Increased salivation. (4) Marked loss of weight. (5) Diarrhoea. The dogs were not killed until severe and unmistakable signs appeared. The normal dogs were divided into Group NC, those just cured of acute blacktongue by a small dose of nicotinamide (2 mg. per kg. body weight daily for 5 days, reckoned on the normal weight of the dog before the onset of blacktongue); and Group N, those whose regimen for some time before they were killed included liberal amounts of nicotinic acid in the diet or large amounts administered subcutaneously or both.

Solutions used for injection were two: a solution of nicotinamide in distilled water, 10 mg. per ml., sterilized by autoclaving; and a solution of nicotinic acid, 100 mg. per ml. made up by adding 4% NaOH solution to solid nicotinic acid until the pH reached 7.4, then diluting to volume with distilled water and autoclaving. The amide had been used in the beginning, but later the sodium nicotinate was substituted in the large doses for economy.

Determination of coenzyme-like substances in blood and tissue extracts. The method used for quantitative estimation of the coenzyme-like substances was that previously described [Kohn, 1938] with the slight modifications mentioned below. It is based on the discovery of Lwoff & Lwoff [1937] that either DPN or TPN can serve as the V-factor essential for the growth of *Haemophilus parainfluenzae*, whereas nicotinic acid or its amide cannot. The method does not distinguish between DPN and TPN, and it is not known whether closely related compounds such as the nicotinamide mononucleotide could serve as V-factor, or whether they occur in the body. For this reason the material assayed will be referred to as V-factor or "coenzyme-like substances". The principle of the method is to determine the quantity of extract necessary to produce an amount of bacterial growth equal to that produced by a standard. We have calibrated our standard against a DPN preparation (65% pure) kindly placed at our disposal by Prof. J. Rnnstrm and Drs A. Lennerstrand and E. Sperber of Stockholm. The results are therefore expressed in equivalent amounts of DPN, or *d.e.* Thus for *blood* an assay of 10 *d.e.* signifies an activity equivalent to 10 μ g. DPN per ml. corpuscles (to which the blood V-factor is confined); for *tissues* an assay of 10 *d.e.* signifies an activity equivalent to 10 μ g. DPN per g. fresh weight of tissue.

The following modifications of the published method have been made. The broth in which the stock cultures were maintained and in which the growth tests were carried out contained in addition to the usual 2% proteose-peptone (Difco brand) and 0.6% NaCl, 0.1% sucrose and 0.04% fumaric acid, titrated with NaOH to pH 7.8 using a glass electrode. The medium was autoclaved no longer than 15 min. at 15 lb. to minimize darkening. The turbidity of the cultures was determined 20–24 hr. after inoculation. A test of the precision of the assay was made by repeating eight times over a period of 1 month a comparative assay of two different standards. The data obtained showed that the standard deviation of this series is $\pm 7\%$, so that the error of a single estimation may be expected to exceed $\pm 7\%$ once in three times and to exceed $\pm 16\%$ once in twenty times.

Preparation of blood extracts. About 0.4 ml. of blood obtained by venepuncture was diluted with 1.6 ml. of oxalated saline solution (3 ml. of 2.2% $K_2C_2O_4$, H_2O + 16 ml. 0.9% NaCl). On one occasion the diluted sample clotted, so a fresh sample was drawn and diluted with a mixture containing a larger proportion of oxalate solution. The haematocrite value of the diluted blood was determined and 0.2 ml. of the diluted blood was laked with 7.8 ml. distilled water and deproteinized by the addition of 2 ml. 2.7% trichloroacetic acid brought to about pH 3 with NaOH. The tube containing the suspension was corked and inverted to sterilize the inside walls, then stored in the cold until required. The tube was centrifuged and the clear extract assayed for V-factor.

Preparation of tissue extracts. The dog was killed by decapitation and the tissues for assay dissected out as rapidly as possible. About 50 mg. fresh weight of the tissue were weighed on a torsion balance and immediately ground with fine sand in the presence of 2 ml. 2.7% trichloroacetic acid (adjusted to pH 2.7–3.0 with NaOH) containing 2% $K_3Fe(CN)_6$. The ferricyanide converts the reduced V-factor into the oxidized form during the extraction; the oxidized form is stable but the reduced form is destroyed on standing at the pH used, therefore the ferricyanide was omitted when it was desired to measure the oxidized V-factor only. After about 30 min. a further 8 ml. of trichloroacetic acid solution without ferricyanide were added and the suspension was transferred from the mortar to a test tube, then corked, inverted to sterilize the tube, and stored in the

cold for several hours. The test tube was then centrifuged and an appropriate quantity of the clear or almost clear supernatant fluid was diluted in sterile broth until 1 ml. of solution contained the extract derived from a quantity of tissue varying from 0.25 to 1.0 mg. according to the tissue and condition of the dog. 0.1, 0.2 and 0.3 ml. of this diluted solution were added to a series of tubes, each containing 7 ml. of broth, which were then inoculated and compared.

The small quantity of tissue used, together with the relatively low pH of the extractive solution permits rapid inactivation of the enzymes which destroy DPN and TPN when the cellular structure is disrupted. In blood the addition of ferricyanide is unnecessary. The tissue extracts have been shown to be stable several days when stored at 1°.

Measurements of O₂ consumption of tissues in vitro. The O₂ uptake of the tissues *in vitro* was measured at 37.5° in the usual Warburg-Barcroft apparatus, filled with O₂. The inner cups contained alkali. After equilibration the O₂ consumption for 30 min. was determined for slices of kidney cortex and of liver, and for strips of thigh muscle, suspended in Ringer phosphate solution at pH 7.4 with 0.1 % added glucose. The Q_{O₂} (ml. O₂ per g. dry wt. per hr.) calculated was designated RG (see results in Table I). Similarly the Q_{O₂} was calculated for slices of cerebral cortex suspended in Ringer phosphate without added glucose and designated R.

After the 30th min. the side arms were tipped, adding to the control vessels 0.5 ml. of Ringer-phosphate, and to the others enough neutralized *dl*-lactate to make a final concentration of 0.024 *M*; except for brain, where enough glucose was added to make a final concentration of 0.2 %. From measurements between the 40th and 70th min. the increment in O₂ consumption due to the addition of lactate or glucose was calculated as Q_{O₂}, designated respectively L and G (see Table I).

Other measurements were made in the presence of added nicotinic acid.

Results

V-factor content of dog blood. Ten dogs were used for this phase of the study, blood samples being taken from 6 to 15 times from each dog over a total period which varied from 61 to 110 days for different animals. Some dogs were kept on the deficient diet until they developed blacktongue, then cured with small doses of nicotinamide, while others were maintained in health on the deficient ration throughout the whole period by daily administration of either a moderate or a large dose of nicotinic acid or its amide. Two dogs were kept throughout on the stock diet, with addition of large daily doses of nicotinic acid for the latter part of the period.

Wide variations in the nicotinic acid intake were found to have little effect on the V-factor content of the blood, which showed no marked change after the development of acute blacktongue. Typical results for three of the dogs are shown in Fig. 1 which indicates the variation in V-factor levels for an individual dog, and also the variation between different dogs. Altogether we have made 15 observations during 11 attacks of blacktongue and 74 observations on normal dogs. The level in the normal dogs varied from 54 to 120 *d.e.* with a mean value of 91 *d.e.* and standard deviation for the series was 15.4 *d.e.* In blacktongue dogs the level varied from 72 to 117 *d.e.* with mean 89.3 *d.e.* and standard deviation of 13.7 *d.e.* The difference between the means is not significant.

V-factor content of dog tissues. As the nicotinic acid intake varied significant changes were found in the V-factor content of liver and of striated muscle

(combined results on diaphragm and thigh) but not of heart, pancreas, kidney or brain. The data are compiled in Table I, which gives individual readings on various tissues from 19 dogs, together with mean values respectively for dogs with blacktongue (Group BT), normal dogs just after cure of blacktongue by small doses (Group NC) and normal dogs on a liberal regimen of nicotinic acid

Fig. 1. Variation in the blood level of V-factor. Dog 4A after a period on the deficient diet developed acute blacktongue on the 1st day recorded and was given 2 mg. nicotinamide per kg. subcutaneously each of the first 5 days. It was cured by the 7th day and relapsed into severe blacktongue again on the 45th day. Dog 93 received the deficient diet + 2 mg. nicotinamide per kg. daily for 18 days before the first reading. This regimen remained unchanged until the 25th day, when the daily dose of nicotinamide was increased to 20 mg. per kg. until the 55th day, after which the nicotinamide was discontinued and 10 ml. crude aqueous liver extract (=300 g. liver) were given daily by mouth.

Dog 16A is included because it was the only dog which showed a marked and sustained change in V-factor level. It was an emaciated but otherwise normal adult dog brought to the laboratory and placed on stock diet 5 days before the first reading. Until the 27th day the diet was unsupplemented, and afterwards 20 mg. nicotinamide per kg. were injected daily until the end. During the period of observation its weight increased from 15 to 19 kg.

Fig. 2. Mean V-factor content of tissues from blacktongue and normal dogs. For each tissue the column at the left is for Group BT, middle for Group NC and right for Group N. The figure at the base of each column gives the number of observations from which the mean was calculated.

(whether on stock diet supplemented by nicotinic acid or amide, or on stock diet with or without supplement) (Group N). They show the wide variability of total V-factor content of most of the tissues for dogs within any one group and similar variability in the proportion of V-factor present in the oxidized form.

Table I

Data on the V-factor content and *in vitro* oxygen consumption of tissues from 19 dogs. Blank spaces indicate that no measurement was made on the appropriate tissue; no readings have been omitted. Dogs in Group BT were killed after development of severe blacktongue on the deficient diet. Dogs in Group NC were killed after cure of blacktongue by administration of an adequate but not excessive amount of nicotinic acid. Dogs in Group C were killed after receiving liberal or large amounts of nicotinic acid. A, B and C had been fed the stock diet for at least 1 month. No. 69 received the stock diet for 3 months and then the deficient diet for 3 weeks. No. 89 received stock diet + 20 mg. nicotinic acid per kg. for 25 days. No. 100 received deficient diet + 2 mg. per kg. for 40 days, then deficient diet + 20 mg. per kg. for 20 days. No. 25 A received deficient diet + 20 mg. per kg. for 20 days. No. 26 A received deficient diet + 20 mg. per kg. for 12 days.

Amounts of V-factor are expressed in d.e. (see text).

Group	Dog no.	Liver				Diaphragm		Thigh				Heart	
		V-factor		Q_{O_2}		V-factor		V-factor		Q_{O_2}		d.e.	
		Total	Oxidized	RG	L	Total	Oxidized	Total	Oxidized	RG	L	Total	Oxidized
Group BT	32 A	165	< 90	—	—	—	—	160	< 80	—	—	—	—
Dogs killed	4 A	90	—	—	—	—	—	385	100	—	—	355	180
during an	98	100	—	9.5	1.4	290	< 80	190	< 80	2.6	0	345	150
attack of	18 A	180	< 60	6.4	0.3	190	100	400	250	2.6	0.1	295	105
blacktongue	12 A	—	155	8.9	0	—	265	—	—	—	—	—	305
	14 A	—	170	10.2	1.1	—	—	—	415	2.8	0.4	—	324
	13 A	—	165	7.3	1.2	—	275	—	—	5.2	0.6	—	260
Group NC	80	285	< 140	—	—	—	—	290	120	—	—	—	—
Normal dogs	24 A	210	< 80	5.2	1.4	345	110	240	185	3.8	0.4	270	255
killed just	7 A	315	158	5.5	1.8	275	145	395	265	2.7	0.5	585	420
after cure of	3 A	125	35	6.0	1.3	205	95	380	295	3.8	0.4	380	315
blacktongue													
Group N	89	355	< 170	—	—	—	—	550	320	—	—	—	—
Other normal	C	460	< 150	—	—	165	—	335	285	—	—	335	135
dogs, see be-	26 A	425	305	6.6	1.6	445	370	360	230	5.2	0.6	420	305
low	100	370	170	6.4	0.9	—	—	495	315	4.2	0.6	410	75
	B	655	380	6.9	0.6	425	405	375	—	4.7	1.1	450	355
	25 A	385	—	—	—	420	255	420	—	—	—	345	240
	69	—	225	6.0	1.3	—	—	—	405	4.3	0.5	—	365
	A	463	273	6.3	1.9	—	—	—	580	4.4	0.5	—	355
Mean of Group BT		134	106	8.5	0.8	240	180	284	175	3.3	0.3	332	220
Mean of Group NC		234	97	5.6	1.5	275	117	326	208	3.4	0.4	412	330
Mean of Group N		445	235	6.4	1.4	364	270	422	363	4.5	0.6	392	296

Group	Dog no.	Pancreas		Kidney				Brain				
		V-factor		V-factor		Q_{O_2}		V-factor		Q_{O_2}		
		Total	Oxidized	Total	Oxidized	RG	L	Total	Oxidized	R	G	L
Group BT	32 A	475	205	—	—	—	—	—	—	—	—	—
Dogs killed	4 A	—	—	660	520	—	—	140	110	—	—	—
during an	98	260	160	490	315	14.8	2.1	115	95	7.7	2.0	3.0
attack of	18 A	270	280	360	235	15.6	1.2	135	90	7.0	3.1	3.8
blacktongue	12 A	—	—	—	340	15.3	1.8	—	105	10.2	3.1	4.6
	14 A	—	320	—	295	18.1	2.6	—	220	8.7	4.4	5.5
	13 A	—	295	—	265	18.4	2.9	—	155	8.2	2.3	2.9
Group NC	80	475	425	—	—	—	—	—	—	—	—	—
Normal dogs	24 A	485	410	520	270	15.3	3.9	85	85	8.8	2.1	4.2
killed just	7 A	360	320	450	340	—	—	104	103	7.9	3.5	4.6
after cure of	3 A	320	—	600	345	15.9	2.7	135	135	8.2	3.7	4.7
blacktongue												
Group N	89	410	390	—	—	—	—	—	—	—	—	—
Other normal	C	430	345	420	350	—	—	155	140	—	—	—
dogs, see be-	26 A	445	390	730	630	14.9	3.9	160	95	8.0	3.7	4.9
low	100	500	380	545	390	15.8	6.2	165	140	9.8	3.0	0.9
	B	340	295	510	265	15.2	4.6	215	205	—	—	—
	25 A	400	345	555	290	—	—	105	65	—	—	—
	69	—	275	—	255	14.8	3.3	—	181	—	—	—
	A	—	275	—	310	16.8	1.9	—	167	—	—	—
Mean of Group BT		335	252	503	328	16.4	2.1	130	129	8.4	3.0	4.0
Mean of Group NC		410	387	523	318	15.6	3.3	108	107	8.3	3.1	4.5
Mean of Group N		421	337	552	355	15.5	4.4	160	142	8.9	3.4	3.8

The tissue in which least variability was found is the heart muscle, which in five dogs of Group N contained from 335 to 450 *d.e.*

The data also show the upward trend of the mean V-factor content for the various tissues from Group BT to Group NC and Group N (see Fig. 2), but this must be interpreted with caution on account of the variation within individual groups. The means in each column of Table I have been compared by statistical analysis of the individual figures in each group, and application of the *t*-test of significance [Fisher, 1936], using the 5 % point as the level of significance (see Table II).

Table II. *Significant differences between Group N and Groups NC and BT*

Tissue	Group	Total V	Respiration
Liver	BT	0.30	1.35 (RG)
	NC	0.55	—
Muscle	BT	0.65	0.75 (RG)*
	NC	0.75*	0.75 (RG)
Kidney	BT	—	0.50 (L)
	NC	—	—

The differences are calculated by comparing the group means at the foot of Table I; Groups BT and NC are expressed as ratios to Group N and each figure is given to the nearest 0.05. A blank indicates that the difference observed was not significant (see text). An asterisk indicates that the difference fell just short of significance, but the presence of a single very discrepant figure in one of the groups compared suggested that additional data would show the difference to be significant.

The figures for V-content of muscle are the combined data of Table I on diaphragm and thigh muscle, but respiration measurements were made only on thigh.

A number of observations which will not be reported in detail showed that in organs excised immediately after the dog was killed about 20 % of the V-factor was destroyed on standing for 1 hr. at room temperature (25°).

The O₂ consumption of tissues in vitro. Changes in the O₂ consumption were observed in those tissues in which the V-factor changed (liver and muscle) and also in kidney, where the V-factor remained steady. These data are shown in Table I. Again all significant differences between means of different groups are collected in Table II. In addition, some observations on the effect of nicotinic acid on O₂ consumption were made. In the presence of 1.3×10^{-3} to 2.7×10^{-3} *M* nicotinic acid at pH 7.4 the RG value for kidney was depressed about 11 % but the increment in uptake produced by lactate was increased about 65 %.

Table III. *Relative amounts of V-factor in various tissues compared with the relative rates of oxygen consumption in vitro and in vivo*

Tissue	V-factor	Respiration	
		<i>In vitro</i>	<i>In vivo</i>
Muscle	1	1	1
Liver	1.1	1.4	1.5
Brain	0.4	2.7	3.0
Pancreas	1.0	—	5.0
Heart	0.95	—	10.5
Kidney	1.3	3.4	10.5

Striated muscle is used as the standard of comparison. *In vitro* respiration was calculated from the data of Table I, using RG for liver, thigh and kidney, and R + G for brain. *In vivo* respiration during maximal activity for brain is based on the reviews of Page [1937] and Quastel [1939]; for other tissues on figures given by Loewy [1925].

The tissues used were obtained from one blacktongue and four normal dogs. No effect was observed with liver or with muscle.

In Table III are given the relative O_2 consumptions of the tissues of normal dogs compared with the relative V-factor contents and with the respiration rates *in vivo* during maximal activity as given by various reviewers. The rate of O_2 consumption per unit amount of V-factor is seen to vary greatly in different tissues. Thus *in vivo* it is from six to ten times greater for heart, brain and kidney than for muscle and liver. *In vitro* brain consumes far more than kidney, muscle and liver. These data show that the relationship between V-factor and O_2 consumption is not simple.

Note on the dry weight of tissues in blacktongue. Since the terminal stages of acute blacktongue are accompanied by dehydration, it appeared possible that our assays of V-factor on tissues of blacktongue dogs might yield figures too high for true comparison with normals on account of loss of water from these tissues. To test this possibility the percentage dry weight of tissues was determined for one dog in each group by drying small samples to constant weight at 110° . The figures are given in Table IV and indicate that there was no increase in the percentage of solids in these tissues in blacktongue.

Table IV. *Dry weight as percentage of fresh weight*

Dog no.	Group	Liver	Kidney	Heart	Pancreas	Brain	Diaphragm	Thigh
18 A	BT	27	22	24	24	—	25	23
24 A	NC	30	24	21	24	19	—	25
26 A	N	31	22	23	33	20	27	—

DISCUSSION

The interpretation of the results given above depends upon the specificity of the V-factor assay. It has already been mentioned that V-factor activity is possessed only by DPN and TPN among known compounds, but there is a possibility that closely related compounds which exert this activity occur unrecognized in tissues. Our mean figures, however, for normal dogs are of the same order as figures reported in the literature for DPN content. These figures, expressed in $\mu\text{g. per g.}$ fresh tissue, are as follows: rabbit skeletal muscle, 400 [Ochoa & Ochoa, 1937]; beef muscle 310, beef liver 700, pig liver 520 [Axelrod & Elvehjem, 1939]; rat muscle, heart, kidney and liver 150–215 [Euler *et al.* 1938]. The percentage of coenzyme in the reduced form varies from 25 to 50 [Ochoa & Ochoa, 1937; Euler & Heiwinkel, 1937; Euler *et al.* 1938]. In rat tissues the amount of TPN is said to be from 15 to 50 % of the amount of DPN [Euler *et al.* 1938]. The older data cannot be reduced to absolute values or else the authors ignore the reduced forms. In view of the lack of evidence to the contrary, and in consideration of the foregoing, we shall assume that the V-factor content is a direct index of the coenzyme content of tissues, either because DPN and TPN are alone in possessing V-factor activity, or because they occur in tissues together with closely related compounds in fairly constant proportions.

Our results as summarized in Table II show that the tissues of the dog may be divided into two classes, those whose V-factor level is influenced by the intake of nicotinic acid, and those whose level is not. To the former belong striated muscle and, particularly, liver. In blacktongue, striated muscle was down to 65 % and liver to 30 % of the value for Group N. When a cure was obtained with an adequate but not excessive dose of nicotinic acid, the fractions rose only to 75 and 55 % respectively and these values were compatible with normal health.

The stability of the V-factor level in brain, kidney, pancreas and blood indicates that some factor other than nicotinic acid intake controls the levels in these tissues. Possibly they are maintained at the expense of liver and striated muscle. The steady rise in the level for dog 16 A (Fig. 1) does not contradict this conclusion. When first brought into the laboratory, the animal was quite emaciated, but showed no signs of blacktongue. For this reason, the subsequent increase in weight accompanied by an increase in blood level should probably be attributed to a lack of some "building stone" other than nicotinic acid.

In man, on the other hand, the administration of nicotinic acid causes a considerable rise in the blood level; in pellagra the latter is somewhat depressed, but on account of the large variations among healthy subjects it remains within normal limits. Thus both in man and the dog, but for quite different reasons, the V-factor level of the blood is of no diagnostic value.

The only observations in the literature are contained in the preliminary statement of Axelrod & Elvehjem [1939]. They compared one deficient dog with normals, and without supplying the data stated that the DPN content of liver decreased by 100 % and of muscle by 1000 % (*sic*). We have found liver to decline more than muscle, a difference which might be attributed to the larger size of our sample or, alternatively, to a much greater decrease in DPN than in V-factor. Their statement that "the cozymase content of the tissues of normal animals remains quite constant" is definitely contrary to our results with liver and muscle.

Only a few data are to be found in the literature on the proportion of tissue coenzymes present in the reduced form (see citations above), and these are for normal animals only. Our observations show variations from no reduced coenzyme-like substance in the brain of each of three dogs of Group NC to a mean of 57 % in the heart muscle of three dogs with blacktongue. There is suggestive evidence that the proportion of reduced coenzyme-like substances is increased with onset of blacktongue in all the tissues examined except heart and kidney where it remains unchanged, and liver for which no comparative figures were obtained.

The changes in V-factor content and O_2 consumption *in vitro* do not run parallel, as shown in Table III. In liver, a 70 % decrease in V-factor was accompanied by a 35 % increase in RG; in striated muscle, a 35 % decrease was accompanied by a 25 % decrease in RG; in kidney, no change in V-factor was accompanied by a 50 % decrease in L. It should be noted that in striated muscle the changes are approximately the same in the BT and NC groups, although the disease exists only in the former. Their presence therefore does not cause the signs of blacktongue.

The relation of these phenomena to the onset of blacktongue and the ultimate death of the dog is obscure, especially since no striking pathological changes peculiar to the disease itself have been described. In acute blacktongue the mucous membranes of the mouth and alimentary tract are invaded by fusospirochetal organs [Smith *et al.* 1937], the dog ceases to eat, and later to drink. There is anuria and often severe diarrhoea and dehydration. These signs, however, are of a secondary nature, and it is to be emphasized that a typical blacktongue syndrome can be produced by a deficiency of vitamin A [Underhill & Mendel, 1928; Smith *et al.* 1937].

The situation may be summarized as follows. The experimental evidence argues against the explanation of blacktongue in the simple terms of a general deficiency of DPN and TPN accompanied by a generalized inability to carry out cellular oxidation-reduction reactions as has been suggested by Euler *et al.* [1939].

On the other hand, the level of these coenzymes is definitely reduced in striated muscle and in liver, and changes in the oxidative metabolism of liver, muscle and kidney can be detected. Of the several possibilities for explanation which suggest themselves, we believe the two following deserve special mention.

(1) When the V-factor level of the liver is reduced below a certain limit, some reaction upon which the maintenance of health depends is impaired because sufficient coenzyme is not available.

(2) Nicotinic acid enters the metabolism in essential ways as yet unknown.

The second possibility has been suggested previously [Daft *et al.* 1938; Kohn, 1938] and has been discussed by Dann [1939], but the evidence is inconclusive, and there is no way at present of favouring either.

It is of interest that these results contrast sharply with those reported for aneurin deficiency where the level of aneurin falls very markedly in brain, heart, muscle and liver, and is accompanied by a decrease in the activity mediated by the coenzyme formed from the vitamin [Ochoa & Peters, 1938; Banga *et al.* 1939; Lu, 1939].

SUMMARY

1. The concentration of coenzyme-like substances (V-factor) has been determined in the brain, heart, striated muscle, liver, kidney, pancreas and blood of dogs (a) with acute blacktongue due to nicotinic acid deficiency, (b) in normal health immediately after cure of blacktongue by small doses of nicotinic acid and (c) in normal health after receiving liberal or large amounts of nicotinic acid. The O_2 consumption of brain, liver, kidney and striated muscle was studied *in vitro*.

2. In dogs with acute blacktongue the concentration of coenzyme-like substances was 70 % lower in the liver and 35 % lower in striated muscle, compared with dogs receiving liberal or large amounts of nicotinic acid. In dogs just cured of blacktongue liver and muscle were 45 and 25 % lower respectively. No difference was found in the other tissues examined.

3. The decreased coenzyme content of the liver in blacktongue was accompanied by an increase of 35 % in O_2 consumption; in kidney the coenzyme content was unchanged but ability to oxidize lactate was decreased by 50 %.

4. In blacktongue the percentage of coenzyme in the reduced form is increased in most tissues except brain and kidney.

5. No evidence was found indicating that blacktongue results from a generalized decrease of coenzyme level in the tissues.

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REFERENCES

- Axelrod & Elvehjem (1939). *Nature, Lond.*, **143**, 281.
Banga, Ochoa & Peters (1939). *Nature, Lond.*, **143**, 764.
Chick, Macrae, Martin & Martin (1938). *Biochem. J.* **32**, 10 and 844.
Daft, Fraser, Sebrell & Pittman (1938). *Science*, **88**, 128.
Dann (1939). *Amer. J. Trop. Med.* **19**, 219.
Dixon (1939). *Ergebn. Enzymforsch.* **8**, 217.
Elvehjem, Madden, Strong & Woolley (1937). *J. Amer. Chem. Soc.* **59**, 1767.
— — — — (1938). *J. biol. Chem.* **123**, 137.

- Euler (1936). *Ergebn. Physiol.* **38**, 1.
— & Heiwinkel (1937). *Naturwissenschaften*, **25**, 269.
— Schlenk, Heiwinkel & Högberg (1938). *Hoppe-Seyl. Z.* **256**, 208.
— — Melzer & Högberg (1939). *Hoppe-Seyl. Z.* **258**, 212.
Fildes (1938). *Brit. J. Exp. Path.* **19**, 239.
Fischer (1939). *Ergebn. Enzymforsch.* **8**, 185.
Fisher (1936). *Statistical Methods for Research Workers*. (Oliver & Boyd, Edinburgh.)
Fouts, Helmer, Lepkovsky & Jukes (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 405.
Funk & Funk (1937). *J. biol. Chem.* **119**, Proc. xxxv.
Green & Brosteaux (1936). *Biochem. J.* **30**, 1489.
Harris (1937). *Nature, Lond.*, **140**, 1070.
— (1938). *Biochem. J.* **32**, 1479.
— (1939). Communication to the Biochem. Soc., 6 May 1939.
Knight (1937). *Biochem. J.* **31**, 731.
Kohn (1938). *Biochem. J.* **32**, 2075.
— & Dann (1939). Communication to the Amer. Physiol. Soc., 28 April 1939.
Koser, Dorfmann & Saunders (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 311.
Loewy (1925). *Handbuch der Biochemie*, ed. C. Oppenheimer, **8**, 1.
Lu (1939). *Biochem. J.* **33**, 774.
Lwoff & Lwoff (1937). *Proc. roy. Soc. B*, **122**, 352 and 360.
Martius (1939). *Ergebn. Enzymforsch.* **8**, 247.
Meyerhof & Ohlmeyer (1937). *Biochem. Z.* **290**, 334.
Mueller (1937). *J. Bact.* **34**, 429.
Ochoa & Ochoa (1937). *Nature, Lond.*, **140**, 1097.
— & Peters (1938). *Biochem. J.* **32**, 1501.
Page (1937). *Chemistry of the Brain*. Baltimore, Md., U.S.A.
Quastel (1939). *Physiol. Rev.* **19**, 135.
— & Wheatley (1938). *Biochem. J.* **32**, 936.
Smith, Ruffin & Smith (1937). *J. Amer. med. Ass.* **109**, 2054.
Underhill & Mendel (1928). *Amer. J. Physiol.* **83**, 589.
Warburg (1938). *Ergebn. Enzymforsch.* **7**, 210.