CXXI. OBSERVATIONS UPON CARBOHYDRATE METABOLISM IN BIRDS.

I. THE RELATION BETWEEN THE LACTIC ACID CONTENT OF THE BRAIN AND THE SYMPTOMS OF OPISTHOTONUS IN RICE-FED PIGEONS.

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Introduction.

The observations embodied in this communication arose in an attempt to discover why some cases of opisthotonus in rice-fed birds could be cured by giving glucose alone [Kinnersley, Peters and Reader, 1928]. This fact, beyond proving incontestably that all symptoms of opisthotonus produced by rice feeding were not due to lack of vitamin B_1, suggested an intimate relation between the symptoms and some phase of carbohydrate metabolism. It seemed possible that investigation of the relation between these symptoms and carbohydrate metabolism might shed light upon the metabolic lesion in avitaminosis proper.

In this paper, it is shown that more lactic acid is present in the brain of a pigeon suffering from "avitaminous" opisthotonus than in that of the normal[1].

Historical.

The brain in avitaminosis B. Though the lactic acid content of the brain in avitaminosis has not been investigated, estimations of other constituents have been made. Schaumann [1910] found only slight differences between the phosphorus content of the brains in normal and rice-fed pigeons. Funk [1912] found a diminution in nitrogen and phosphorus and Findlay [1921] a decrease in nucleic acid content, whereas Hotta [1923] and Verzar, Kokas and Arvay [1924] claimed an increase in cholesterol content, and more recently, Ljubarskaja [1928] an increase in creatine.

[1] We have purposely avoided using the term "beriberi pigeon" throughout owing to the claim by McCarrison [1928] that true beriberi can be produced in pigeons, and that this differs from simple polyneuritis. A preliminary account of the work appeared in *Chem. Ind.* (1929), **48**, 360.

The lactic acid content of brain tissue. Several workers have made estimations of the lactic acid content of the brain in other animals. McGinty and Gesell [1925] found that it varied in the brains of rabbits and dogs according to the time of removal after death. The lowest value recorded by them for rabbit brain was 43 mg. per 100 g. They concluded that 60 mg. of lactic acid per 100 g. accumulated during the first 3 minutes. In anoxaemia the amount of lactic acid found was much larger. The average value for dogs' brains put into liquid air within 7–45 seconds was 70–80 mg. per 100 g.

About the same time Warburg, Posener and Negelein [1924] and Loebel [1925] showed that sections of the grey matter of the brain of the rat were actively glycolytic, and Holmes and Holmes [1925, 1926, 1927] showed that the lactic acid content of rabbit brain within some 3·5 minutes from death varied as the sugar content of the blood. This value in effect represents the total lactic acid which can be formed from carbohydrate precursors in the tissues, as it does not increase upon incubation in phosphate-buffer solution. The lowest value for brain-lactic acid obtained by Holmes and Holmes was 20–35 mg. per 100 g. in an insulinised animal. They did not consider the changes to be correlated with the lactic acid content of the blood. Haldi, Ward and Loo [1927] found that the amount of lactic acid differed in different parts of the brain. These authors used a liquid air technique and obtained remarkably low values for the lactic acid content, in the case of the cerebral hemispheres 11·6–14·7 mg. per 100 g. McGinty [1929] has claimed from the analysis of the lactic acid content of the blood entering and leaving the brain that there is a lactic acid metabolism of the tissue similar to that found in other tissues. The general conclusions to be drawn from the above are that there is probably some lactic acid present in the brain during life but that the actual value is obscure owing to the very active glycolytic powers of the nervous tissues.

The blood. Estimations of lactic acid in the blood of pigeons have been performed by Collazo and Morelli [1925], who found for the normal pigeon 29 mg. and for three cases of avitaminosis 54–57 mg. per 100 g. It is not clear from the protocol whether the three birds had symptoms at the time of estimation or not.

Experimental.

General methods. We have employed, for the estimation of lactic acid, the method of Friedemann, Cotonio and Shaffer [1927], and for the estimation of reducing substances, Hagedorn and Jensen's [1923] method. For deproteinisation we have employed 10 % trichloroacetic acid, and for removal of sugar the copper-lime precipitation. Estimations have been performed upon the brains of normal and rice-fed pigeons at various stages of avitaminosis. From preliminary experiments we learnt the importance of avoiding asphyxia, with its consequent effect upon blood-sugar, and of early stoppage of the supply of blood to the head. We therefore stunned the animal, cut off the head and rapidly removed the brain, which was immersed in ice-cold 10 % tri-

chloroacetic acid [Holmes and Holmes, 1925] as soon as possible after death. The values so obtained were much lower than those of our preliminary experiments. Table I shows there is still present in such brains precursors capable of conversion to lactic acid upon incubation in phosphate-Ringer's solution (p_H 7·6).

Table I. *Effect of incubation upon the brain-lactic acid.*

Animals stunned, brains rapidly removed; half was ground immediately in ice-cold trichloroacetic acid, remainder incubated at 37° for 60 min. in phosphate-Ringer (p_H 7·6) before grinding with acid.

Exp.	Time after stunning sec.	Brain		Brain after incubation	
		Lactic acid*	Sugar	Lactic acid*	Sugar
1	45	95	125	124	64
2	60	95	92	107	73
3	90	110	77	133	58

Values = mg. per 100 g. brain.
* Lactic acid single estimations.

These results suggested that any change of consequence probably occurred within the first 45 sec. after stunning. Accordingly we next used liquid air, and worked as rapidly as possible. The bird was stunned by hitting its head upon the table, the head removed and the brain then plunged into liquid air immediately.

Freezing the whole head gave no lower results for the lactic acid and was attended with the error consequent upon the presence of pieces of other tissues, as it was found impossible to separate the frozen brain tissue properly. This technique gave us the lowest results which we have obtained.

Description of an experiment. The pigeon was stunned and the head cut off. The brain was frozen in liquid air, and the blood collected into oxalate from the neck. The cerebral hemisphere and part of the mid-brain were immersed in 20–25 sec. from the time of stunning, and the remainder within 30–40 sec. The frozen pieces of brain weighing 1–2 g. were placed into 10 cc. of ice-cold 10 % trichloroacetic acid in a tared, corked 25 cc. centrifuge tube. The tube was immediately re-weighed, and the tissue broken up in the ice-cold trichloroacetic acid. After standing 15–30 min. with occasional stirring, the tube was centrifuged and the supernatant liquid decanted into a similar centrifuge tube. Two cc. of 10 % copper sulphate and 5 cc. of a 5 % suspension of calcium hydroxide were added, and allowed to stand for at least half an hour with stirring. The tube was then centrifuged, the volume measured and the fluid decanted into a distillation flask, to which had been added powdered talc, 10 cc. of 28·5 % sulphuric acid, and 10 % manganous sulphate solution, and made up to approximately 100 cc. with water. The remainder of the estimation was carried out in accordance with the technique of Friedemann, Cotonio and Shaffer [1927]. In the earlier experiments in making the calculation it was assumed that the lactic acid in the original extraction with 10 % trichloroacetic acid was distributed evenly between the precipitate and the

filtrate. This was confirmed in two experiments. A similar assumption was made in the case of copper-lime precipitation. In the later experiments, two extractions with trichloroacetic acid were made. The results did not differ appreciably.

0·1 cc. samples of the blood were taken for direct estimation by the Hagedorn and Jensen method. The remainder was treated with water in the proportion of 8 cc. water and 1 cc. 25 % trichloroacetic acid to 1 cc. blood. This was stirred and allowed to stand before centrifuging. The reducing value of the trichloroacetic acid filtrate from the brain was determined in most cases upon an aliquot sample (1/20) of the original 10 % trichloroacetic acid filtrate.

The probable accuracy of the results. Care has been taken to standardise the procedure so that the results are strictly comparable. The estimations are subject to the usual errors involved in the handling of small amounts of tissue. Apart from these, the sugar values quoted are the mean of duplicate determinations and may be considered reliable to 3 mg. sugar per 100 g. (or cc.). The lactic acid values for blood, unless otherwise stated, are the mean of duplicate estimations, and may be relied upon to 1 mg. lactic acid per 100 cc. The lactic acid values for brain in the earlier results represent one estimation upon the whole brain in order to reduce to a minimum the errors of handling small amounts of tissue. Many of the later results have been performed in duplicate. In all cases, the differences upon which the conclusions have been based are too large to be influenced appreciably by the errors of the methods.

The question of cold extraction. Though care has been taken throughout to immerse the frozen brains in thoroughly cooled trichloroacetic acid, and to grind them in this while cold, we have not found appreciably higher results even if the tissue is allowed to return to room temperature in the acid before extracting. It would therefore appear that the glycolytic system of brain tissue is completely disintegrated by freezing in liquid air. In this it resembles blood [Irving, 1926] but apparently not muscle [Fletcher, 1913; Macleod, 1928].

The nature of the substances estimated. (*a*) The reducing values obtained by the Hagedorn and Jensen method have been described throughout this paper as "sugar." We do not wish this to be regarded as an indication that we believe them to represent glucose only. In the case of the brain, it is known from the work of Holmes and Holmes and also of Ljubarskaja that considerable amounts of creatine are present in addition to any actual glucose. In work performed in collaboration with Dr J. M. Gulland, evidence has been obtained that other substances than glucose are present in the blood [cf. Herbert and Groen, 1929].

(*b*) The same argument must be applied to the values represented as "lactic acid," since this has not actually been isolated, although there is indirect evidence that it is mainly lactic acid which is so estimated. The filtrates even from the avitaminous birds do not give a nitroprusside reaction.

McGinty and Gesell proved that in brain from the rabbit much of the substance which they were estimating was lactic acid, and the method of Friedemann, Cotonio and Shaffer is known to be more specific than other methods which have hitherto been used.

The brain. No attempt has been made in this work to differentiate between different parts of the brain, though we realise that such a treatment of so highly organised a tissue is grossly inadequate. There is evidence to show that different parts of the brain differ in lactic acid content [Haldi, Ward and Loo, 1927]. Further we ourselves have found, in cats' brains, the lactic acid content of the grey matter to be some 30 % more than that of the white, a difference which Prof. J. Mellanby told us he had observed some years ago.

Table II gives results for normal pigeons and pigeons suffering from opisthotonus due to lack of vitamin B_1. Parallel estimations have been performed upon the brain and blood. The avitaminous pigeons have been obtained by feeding upon polished rice as described by Kinnersley, Peters and Reader [1928].

Table II. *Comparison of brain- and blood-lactic acid (liquid air technique).*

"Time" represents the time after stunning and before immersion in liquid air. Values represent mg. per 100 g. tissue.

		Brain		Blood	
Exp.	Time sec.	Lactic acid	Sugar	Lactic acid	Sugar
Normal pigeons.					
4*	? 10	92	—	21	246
5	30–35	87	—	19	242
6	20–30	82	87·5	20	191
7	20–40	94†	109	45†	166
8	20–30	74	100	24	226
9	25–35	79	110	23	232
10	25–40	93	107	26	226
11	30–45	78	93	29	285
Opisthotonus pigeons.					
12*	15	109	95·4	—	219
13	20–30	112	93·5	—	281
14	30–35	114	109	44	250
15	20–30	105	117	40	212
16	20–40	133	143	57	281
17	17–30	105	133	122	306
18	30	134	102	60	225

* Head immersed whole, a procedure discontinued in subsequent experiments.
† Excluded from average. Bird abnormal, both in low blood-sugar and high lactic acid.
Lactic acid values for brain 1 estimation, lactic acid values for blood mean of 2 estimations except in Exps. 11, 15, 17.

In further experiments we have found that provided care be taken to anaesthetise without disturbance, the blood-sugar is not raised. Table III shows some observations upon birds under ether and chloroform. It will be seen that the lactic acid value is not raised in the brain 10–20 sec. after death, nor as a general rule in the blood, as the result of simple anaesthesia.

Tables II and III show that there is much more lactic acid in the "avitaminous" brain than in the normal. Before any conclusions can be drawn from this, however, certain other possibilities must be considered.

Table III. *Effect of anaesthetics upon normal birds.*

Mg. per 100 g. tissue.

Exp.	Anaesthetic	Time sec.	Brain		Blood	
			Lactic acid	Sugar	Lactic acid	Sugar
19*	Ether	—	65	125	12·5	212
20	,,	10–15	50	120†	10	192
	,,	60	100	122‡	10	192
21	,,	10–40	60	117	28	240
22	,,	15–23	53	136	18	230
23	,,	10–20	53	137	14	219
24	Chloroform	10–15	64	130	20·5	214
25	,,	8–25	64	105	20·5	217

* Brain exposed during anaesthesia, and frozen *in situ*.
† Upper parts of brain removed rapidly.
‡ Lower half of same brain allowed to stand after cutting off circulation.
Lactic acid values: for blood, duplicate estimations, except in Exp. 24; for brain, duplicate estimations in Exps. 20, 22, 23, 25; remainder, single estimations.

The variations in the results and the gradual reduction in the amount of lactic acid as the technique has improved, indicate that none of the values represents the percentage amount of lactic acid at the instant of death. The question therefore arises as to what proportion of the lactic acid found has arisen by a rapid *post-mortem* glycolysis. In fact, more explanations than one are possible to account for the increased lactic acid: either (1) there is more lactic acid present at death; or (2) there is no more lactic acid than normal present at the instant of death, but more lactic acid has arisen *post-mortem* owing to either (*a*) an increased blood-sugar, or (*b*) an enhanced glycolytic power of the tissue in the abnormal brain. That the increased lactic acid is not due to raised blood-sugar is proved by Exps. 12, 15, 18 in which the blood-sugar was not higher than the normal. This disposes of the supposition in 2 *a*; 2 *b* is, however, more difficult to meet. If the abnormal brain has a more active glycolytic system, then estimations at different time intervals after death should reveal it. A T-shaped guillotine was constructed for the pigeon [cf. McGinty and Gesell, 1925], and so arranged that the knife which severed the neck operated before that which split the skull. Thus the circulation was interrupted before cutting the brain, which we believe to be important. Though the total amount of tissue in the brain of the pigeon is small, this very fact ensures that the pieces removed are rapidly cooled. Owing to the results of Table III, and to facilitate rapid manipulation, ether was used in many of the experiments. The results upon separate halves of brain obtained with the guillotine are given in Table IV. The halves were scooped from the skull into liquid air, the second half being allowed to stand for a short interval in air before freezing.

The experiments show that even within 12–15 sec. of death as much as 58 mg. lactic acid per 100 g. tissue is present in the brain. Thus the improved method has not reduced the lactic acid values. By standing for periods up to 1 min. about 20 mg. more lactic acid per 100 g. tissue accumulates. If we assume that the normal brain tissues at death contain about 15 mg. lactic

Table IV. *Separate halves of brain.*

Mg. per 100 g. tissue.

Condition	Exp.	Method of killing	Time* (sec.) 1st half	Time* (sec.) 2nd half	Brain: Lactic acid 1st half	Brain: Lactic acid 2nd half	Brain: Sugar 1st half	Brain: Sugar 2nd half	Blood: Lactic acid	Blood: Sugar
Normal	26	Stunning	12	40	72	71	110	124	—	—
	27	„	15	105	66	—	150	152	48†	222
	28	„	17	50	60	79	123	151	19	219
	29	Ether	17	65	59	81	175	180	15	219
	30	„	12–25	65–90	58	87	164	125	16	233
	31	„	15	90	60	88	158	116	19†	212
Opisthotonus	32	„	13	80	91	156	189	175	48	212
	33	„	15	100	100	154	141	97	60	205
	34	„	11	60	134	147	239	163	153	255

* Time at which the half-brain was immersed in liquid air.

† All values the mean of duplicate estimations, except those marked with an obelus (†).

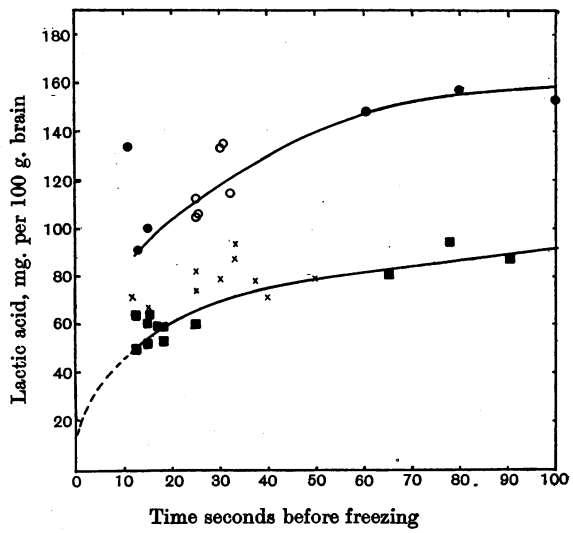

Fig. 1. Rate of appearance of lactic acid in pigeons' brains after death.

●=Anaesthetised opisthotonus. ○=Stunned opisthotonus.

■=Anaesthetised normal. ×=Stunned normal.

Upper curve anaesthetised opisthotonus. Lower curve anaesthetised normal.

(Curve drawn through points from Table IV. Other points from Tables I, II and III.)

acid per 100 g. (the amount found for the blood-lactic acid), then the curve drawn in Fig. 1 will represent the approximate rate of evolution of lactic acid in the normal brain. Turning now to the opisthotonus results, if we leave out Exp. 34, in which there is a very large amount of lactic acid present even in 11 sec., in Exp. 32, 91 mg. of lactic acid were found, *i.e.* 30 mg. more lactic acid were present in this case than in the normal. If the brain-lactic acid at death in this case of opisthotonus was no more than the normal, *i.e.* 15–20 mg., then we should have to believe that the glycolytic system was twice as active

as the normal, and in Exp. 34 even more active. Such an assumption seems to be improbable. It is, moreover, not supported by some earlier experiments in which normal and "avitaminous" brains were incubated with buffer solution with and without glucose. The experiments gave no evidence of enhanced glycolytic activity.

The conclusion reached, therefore, is that actually more lactic acid is present at death in the "avitaminous" brain. The results in Table IV suggest other points of general interest, though subsidiary to the present investigation.

In Fig. 1, points from Tables I, II, III have been added. It will be seen that stunning gives more rapidly a higher yield of lactic acid than does anaesthesia. This suggests that much of the lactic acid found is produced instantaneously by the trauma. It is possible that the severing of the neck produces lactic acid by stimulation, and that in anaesthesia such stimulation is diminished. The similarity implied between the processes of muscular activity and those of the central nervous system is clearly very close.

It is remarkable that the lactic acid formed stops short at a maximum much below that produced on incubation for an hour in phosphate-Ringer's solution. More lactic acid-forming material is present in the avitaminous brain (Exp. 32). The matter lies outside the scope of the present investigation, but the following facts may be noted. The "sugar" values for the brain filtrates, unlike the lactic acid values, show no consistency, though there is a general tendency for an increase with a high blood-sugar. As part of the reduction is due to creatine (Holmes and Holmes) the figures cannot be interpreted in detail without special investigation; the failure, however, to account for the increase in lactic acid at the expense of reducing substances in Exps. 29 and 32, for instance, suggests that the lactic acid arises from some non-reducing carbohydrate.

It is to be noted that with few exceptions the normal blood-lactic acid has lain between 14 and 29 mg. per 100 cc. with an average value of 19 mg. This is rather lower than the value given by Collazo and Morelli [1925]. Avitaminous pigeons have given considerably higher values (40–150 mg.). In confirmation therefore of these authors, birds with symptoms have a raised blood-lactic acid.

It may be asked next whether the appearance of increased lactic acid in the brain is a necessary concomitant of (*a*) these symptoms, and (*b*) of all similar symptoms.

This evidence is difficult to obtain directly, and has been sought for along two paths. Firstly, is there evidence that the increased lactic acid appears only when the convulsions appear, and that it disappears rapidly upon cure of the condition? Secondly, are convulsions of the opisthotonous type always associated with an increased brain-lactic acid. In the experiments of Holmes and Holmes [1926], insulin convulsions in the rabbit were associated with a low lactic acid in the brain. The first question is dealt with in Table V. The second will form part of a subsequent communication.

Table V shows that birds which had been fed for many days with rice,

even with blood-lactic acid as high as 28–30 mg. per 100 cc., did not have a high brain-lactic acid except in Exp. 40, where the bird had been longer upon the diet, and therefore was undoubtedly nearer to the symptoms than the others. The fact that a bird may be sufficiently ill to have a raised content of lactic acid, Exp. 36, in the blood without showing a high brain-lactic acid seems to us to be indirect evidence in favour of the view which is here put forward. After the cure of the symptoms three birds showed a normal lactic acid content in the brain though in one (Exp. 41) not completely so in the blood. It may be concluded provisionally that there is less lactic acid present in the brain both before and after the cure of the symptoms than during them, and that the lactic acid in the blood returns more slowly to the normal than in the brain. The general conclusion therefore is that increased lactic acid in the brain is a necessary accompaniment of opisthotonus produced by lack of vitamin B_1.

Table V. *Relation of lactic acid to the presence of symptoms.*

Mg. per 100 g. (cc.).

(*a*) Before the appearance of symptoms*.

Exp.	Days of rice feeding	Time sec.	Brain		Blood	
			Lactic acid	Sugar	Lactic acid	Sugar
35	10	25–35	76	113	16·5	226
36	22	18–25	89	122	30	203
37	22	18–25	81	112	23	245
38	23	20–30	65	92	28	236
39	23	40	86	111	17·5	215
40	27	22–30	116	131	32	231
		(*b*) After curing the symptoms†.				
	Hours after cure					
41	4·5	20–30	57	124	28	239
42	12	13–20	70	105	21	217
43	2	13–20	56	118	21·5	200

* Single estimations of lactic acid, except in case of Exps. 35, 36, 37 (blood-lactic acid).
† Duplicate estimations throughout.

Discussion.

It is evident that our experiments lend considerable support to the views of Bickel [1924 1925] and Collazo [1922] on the significance of increased lactic acid in the blood and urine in vitamin B deficiency, and on their connection with derangement of the carbohydrate metabolism. We do not accept, of course, the view that the symptoms of opisthotonus are a mere incident, as we have found that they tend to appear at a constant time in the same bird [Kinnersley, Peters and Reader, 1928], and to behave in a standard way to doses of a curative extract. Funk and v. Schönborn [1914] found hyperglycaemia in rice-fed pigeons; Collazo [1922] described a stage of hypoglycaemia followed by hyperglycaemia, together with carbohydrate intolerance. The blood-sugar, after a dose of sugar by mouth, remained higher than normal. Prof. H. M. Evans has told us that he and his colleagues have found

carbohydrate intolerance in vitamin B-deficient rats. Our normal birds have a blood-sugar from 190–220 mg. per 100 cc.; a few have been found with higher values. In rice-fed birds the values have tended to be 10 mg. higher. Birds with symptoms have fallen into two groups—some have normal blood-sugar, others 280–320 mg. per 100 cc. This occurs especially in birds which have been dosed with glucose within the last few hours, suggesting carbohydrate intolerance in confirmation of the results of the other workers. The hyperglycaemia observed by others is therefore due to forcible feeding [Randoin and Lelesz, 1926]. Our birds are always allowed to feed naturally. It is held by some that such birds are suffering from avitaminosis plus inanition. That this does not interfere with the response to torulin is conclusively shown in this paper and elsewhere. No question of an absolute lack of available food material or of ions to supply essential processes can arise, when 1 mg. or less of a torulin preparation suffices to cure the condition, and render the animal again able to feed (Table V). The argument that, because by forcible feeding it is possible to maintain the weight of an animal, it is not suffering from inanition and is therefore a less complicated subject for experiment when avitaminosis arises, is not without fallacy. A forcibly fed bird suffering from lack of vitamin B_1 has a permanent hyperglycaemia, so that the tissue cells are being continually bathed with an abnormal concentration of blood-sugar. The animal which feeds normally doubtless does not eat so much in the later stages and is often found to have a practically normal blood-sugar. It is not improbable that the lack of appetite is correlated in some degree with an adjustment of the blood-sugar.

The evidence in this paper throws open again the question whether storage carbohydrate exists in the brain tissue. Holmes and Holmes advanced the view that the lactic acid of the brain had its origin in the blood-sugar. Their values however were lactic acid "maxima." Our experiments seem to us to leave no doubt that lactic acid originates from some form of carbohydrate in the brain itself. The amount of lactic acid which arises in some cases is more than could be produced by blood present in the brain even considering that such occupied one-tenth of the volume of the brain tissue.

Summary.

1. Pigeons in the terminal stages of avitaminosis B_1, showing symptoms of opisthotonus, have an increased amount of lactic acid in the brain as compared with the normal. Fifteen seconds after death there is found in the normal approximately 55 mg. per 100 g. tissue, and in the avitaminous 95 mg.

2. The increased lactic acid is not a consequence of the high blood-sugar, and tends to follow the blood-lactic acid, which is raised. At the end of a period of 40 sec. after death about 70 % of the lactic acid maximum is formed in the brain. Stunning produces lactic acid more rapidly than careful anaesthesia with ether.

3. The increased lactic acid is a concomitant of the symptoms in avitaminosis B_1, and disappears within a short period after dosing with torulin.

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