CCLIX. URIC ACID SYNTHESIS IN PIGEONS. II.

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THE demonstration by von Knierem [1877] and von Schroeder [1878] that the nitrogen of amino-acids and of ammonium salts can be converted into uric acid in the organism of the bird, and Minkowski's [1886] demonstration that after hepatectomy in the goose the uric acid content of the urine fell to a very low value and large amounts of dextrorotatory lactate were excreted, together with the tacit assumption of an underlying similarity in the processes of ammonia detoxication in the bird and mammal, ultimately led to the attractive hypothesis of the formation of uric acid by the condensation of one molecule of lactic acid, or of a related compound, with two urea molecules.

Most investigations of this synthesis have been based on this hypothesis. For instance Wiener [1902] believed that he could show that a very large number of compounds related to lactic acid would produce an increase in the uric acid excretion of hens given large daily doses of urea, up to 100 % of the compound administered being recovered as uric acid. However, a statistical examination of some of Wiener's results renders his conclusions very doubtful (see the Appendix to this paper). Again Ascoli and his colleagues [Ascoli, 1898; Ascoli and Izar, 1909; Bezzola *et al.*, 1909; Izar, 1910, 1, 2; 1911; Preti, 1909], in an elaborate series of investigations, claim to have demonstrated uric acid synthesis from non-purine precursors in mammalian and avian livers and have also claimed that by their technique the synthesis of uric acid from dialuric acid and urea can be demonstrated in liver "brei". But attempts to repeat their fundamental observations made by Spiers [1915] and by Calvery [1927] have failed.

Pupilli [1928; 1930] has claimed recently that in the hen urea is conjugated with most of the compounds used by Wiener to yield uric acid, and that the same process can be observed in liver powder. However, he relies on an iodimetric method of estimating uric acid due to Ganassini [1914], which does not seem to be very specific, and his results are at variance with those of Clementi [1930], who claims that injected urea can be recovered quantitatively from the excreta of birds, whether or no salts of such acids as pyruvic and malonic acid are simultaneously administered. Torrisi and Torrisi [1931] and Russo and Cuscunà [1931] have published work in support of Clementi's conclusions. Torrisi and Torrisi appear to show, in addition, that the administration of pyruvic and malonic acids has no effect on uric acid excretion by the hen.

Three investigations which do not rest on the original hypothesis show considerable accord with one another. Kowaleski and Salaskin [1901] claimed that they had shown that when a goose liver was perfused with blood uric acid was added to the blood, and that the extent of this addition was greatly increased if ammonium lactate were added, a finding which appears to indicate, in view of the continued failure to obtain urea synthesis in isolated perfused liver preparations, that urea is not a precursor of uric acid. Schuler and Reindel [1933, 1, 2] and Krebs and Benzinger [1933] have shown, independently, that the

kidney, as well as the liver, may play an important part in the synthesis of uric acid in some birds, and the latter workers, by utilising this discovery, have been able to show convincingly that ammonia can act as a source of nitrogen for uric acid synthesis and that urea cannot.

Krebs and Benzinger, working with tissue slices, took the opportunity of testing a number of compounds as sources of carbon for the synthesis. Of those tested, only *dl*-lactate, pyruvate and glucose increased the rate of uric acid synthesis of their preparation, and these increases in rate were observed only when tissues from a starving animal were used. In view of the observation of Krebs and Henseleit [1933] that the same substances increased the rate of urea synthesis in mammalian liver slices, *i.e.* accelerated a synthesis in which they did not participate, it seemed very likely that, in both instances, the acceleration occurred by way of an improvement in the nutritional state of the tissue.

Whereas, then, it is reasonably certain that the source of the nitrogen for the synthesis is ammonia, and that urea is not an intermediary, there is very little positive information concerning the source of carbon, as the following table shows:

Reputed sources of carbon for uric acid synthesis.

Source	Investigators			
	W	TT	P	KB
<i>dl</i> -Lactic acid	+	.	+	?
<i>l</i> -(+)-Lactic acid	+	.	.	.
Pyruvic acid	+	-	.	?
Tartronic acid	+	.	+	-
Glyceric acid	(+)	.	+	.
Hydraacrylic acid	+	.	.	.
Mesoxalic acid	+	.	-	-
Formylglyoxylic acid	.	.	+	.
Glycerol	+	.	.	.
Glucose	.	.	.	?
Propionic acid	-	.	.	.
Malonic acid	+	-	-	-

W = Wiener
 TT = Torrisi and Torrisi
 P = Pupilli
 KB = Krebs and Benzinger

+ = increased uric acid synthesis
 (+) = doubtful increase
 ? = increase, but evidence against participation
 - = no increase

Wiener's results are statistically unsound, as I shall show later. Neglecting them, it will be seen that, in every instance but one in which more than one investigation has been made, there is disagreement as to the ability of the substance in question to increase uric acid production. The one instance is that of racemic lactate, and it seems therefore that the most important question remaining to be answered in connection with this synthesis is: does lactic acid stimulate the synthetic mechanism or does it participate directly in the synthesis?

The investigation to be described in this paper is an attempt to answer this question. The general plan of the investigation is that the relation between food intake and uric acid excretion of a pigeon is determined, as described in the foregoing paper [Fisher, R. B., 1935], and then the same relation is redetermined whilst the pigeon is receiving a constant daily dose of sodium lactate. As there is no restriction on the amount of food eaten by the pigeon, and as the body weight remains constant within very narrow limits, there is no reason to suppose that the lactate administered can improve the nutritional state of any tissue. Further, the lactate administered has never been more than the equivalent of 100 mg. of the free acid *per diem*, so that its energy equivalent has not exceeded approximately 360 g. cal. On the basis of Benedict and Riddle's [1929] figures, the

birds used in these experiments should dissipate between 50,000 and 70,000 g. cals. *per diem*, so that the lactate fed could supply at most 0.7 % of the bird's daily energy requirement.

Since, however, the base fed with the lactate may produce an effect similar to that which it would produce in a mammal, namely, sparing action on ammonia production by the kidney, it is possible that nitrogen will be made more readily available for further uric acid synthesis. This point can be tested by feeding equivalent amounts of sodium bicarbonate or sodium acetate, but a more elegant and more complete control would be obtained if it could be shown that one of the optical isomerides of lactic acid, fed as the sodium salt, increased uric acid excretion, whereas the other did not do so.

Experiments of this type have been performed, in which one of a pair of birds received sodium *l*-(+)-lactate and the other received sodium *d*-(-)-lactate. A period of twelve days was arbitrarily chosen for the lactate administration. It seemed possible that a gradual adaptation of carbohydrate metabolic mechanisms might take place during prolonged administration of lactate, and it was decided therefore that the period of lactate administration should be as short as was consistent with the establishment during the period of a definite regression of uric acid excretion on food intake. The lactate was given by pipette once every 24 hours, at the end of the feeding period, and the excreta were analysed in 48-hour samples as in the previous work.

The lactate solutions used were prepared from the zinc ammonium salts, and the final solutions were examined polarimetrically and tested for the presence of ammonia. In no instance was racemisation detected or ammonia found in the solution.

Typical results are presented in Figs. 1-4. The hollow circles represent results obtained without lactate feeding: the full circles represent results obtained during lactate feeding. It is obvious that in Figs. 1 and 3 the uric acid excretion at a given food intake is constantly above the normal level, whereas in Figs. 2 and 4 there is no constant difference between normal and lactate results.

Since it is also obvious that the differences between the results obtained in normal periods and during lactate feeding cannot be expressed simply, it is necessary to consider at this point the probable nature of the different possible actions of lactate on uric acid excretion.

In the first place, it has been shown that *l*-(+)-lactate increases the uric acid excretion at a given food intake, whereas *d*-(-)-lactate does not, so that it is no longer necessary to consider the possibility that the effect of lactate is due to the base administered. (There is no reason to suppose that the birds would have any difficulty in metabolising completely the amounts of *d*-(-)-lactate administered, since these never exceeded 100 mg. of the free acid *per diem*.)

The possibilities left are (1) a specific stimulant action of lactate on the synthetic mechanism, and (2) a participation of lactate in the synthesis. Other possibilities, such as alterations of excretion of uric acid rather than its synthesis, are rendered unlikely by the persistence of the effect over a period of twelve days.

In view of the fact that the relation between nitrogen intake and uric acid excretion is linear (see previous paper), it is unlikely that the synthetic mechanism is saturated with respect to nitrogen. Yet only about 50 % of exogenous nitrogen is excreted as uric acid. Many types of explanation of this fact might be devised, but it is at least possible that this failure to convert more nitrogen into uric acid is in some measure due to inadequate supply of the source of carbon. If we consider the possibility that the source of carbon for the synthesis may be com-

Fig. 1.

Fig. 1. Effect of *l*-(+)-lactate. Bird 617. 100 mg. of *l*-(+)-lactic acid (as the sodium salt) per 48 hours.

Fig. 2.

Fig. 2. Effect of *d*-(-)-lactate. Bird 617. 150 mg. of *d*-(-)-lactic acid (as the sodium salt) per 48 hours.

Fig. 3.

Fig. 3. Effect of *l*-(+)-lactate. Bird 617. 200 mg. of *l*-(+)-lactic acid (as the sodium salt) per 48 hours.

Fig. 4.

Fig. 4. Effect of *d*-(-)-lactate. Bird 2000. 200 mg. of *d*-(-)-lactic acid (as the sodium salt) per 48 hours.

peted for by the synthetic mechanism and by some other metabolic mechanism which need not be specified, then it is easy to see how, in normal circumstances, the extent of the conversion of exogenous nitrogen into uric acid may be so small. If in these circumstances the synthetic mechanism is activated by some regularly presented stimulant, then the proportion of nitrogen presented to it which will be converted into uric acid will be increased. On the same hypothesis, if an excess of the source of carbon be regularly presented in such a way that the relative activities of the synthetic and competing mechanisms are unaltered, then a constant excess of uric acid over the normal for the particular food intake should be excreted, and this amount should never reach the theoretical maximum calculated on the basis of the complete conversion of the administered substance.

In other words, if

$$Y = bx + A$$

represents the relation between food intake and uric acid intake, where Y = food intake per 48 hours, x = uric acid excretion per 48 hours, and b and A are constants, it follows that the administration of lactate, if it stimulate the synthetic mechanism, will bring about an increase in b , whilst if it participate in the synthesis it will bring about an increase in A .

From these considerations it is obvious that the differences between the normal and lactate results shown in Figs. 1-4 can best be expressed in terms of the parameters of the regression lines. Since the samples of data available are small, and since the estimates of those parameters which are obtained will be approximations, the following procedure has been used to determine how far the observed differences are significant ones.

In the first place the parameters of the regression lines for the series of data obtained during normal feeding and for the series obtained during lactate administration have been calculated for each bird. In the instance of the data of Fig. 1 the regression lines are:

$$Y = 17.6988x - 4.34 \dots \text{normal}$$

$$Y = 21.2925x - 48.51 \dots \text{lactate.}$$

The standard error of the difference between the two estimates of b can be calculated [R. A. Fisher, 1932, p. 126]. In this instance it is 8.272. The ratio of the observed difference between the two estimates of b to the standard error of that difference (*i.e.* here $(21.2925 - 17.6988)/8.272 = 0.511/1$) constitutes the statistic t . It is possible to read off from the appropriate tables [R. A. Fisher, 1932, p. 151] the probability that, in two such samples as these, a difference, of the magnitude observed, between the values of b could arise by chance even if the samples were random ones from the same population. The less the probability of such a difference, the greater the value of t .

In this instance the value of t is 0.511. For a set of data possessing, as this one does, 21 degrees of freedom, $t = 0.532$ when $P = 0.6$. In other words there are rather more than six chances out of ten of obtaining differences in b of the observed magnitude between two series of data from the same normal bird, so that lactate administration cannot be said to have influenced the value of b significantly.

The next step is to determine the significance of the differences between the values of A . The first step, since we are assuming the values of b to be the same within experimental limits, is to obtain a pooled estimate of b from the two series of data and to calculate corresponding new values of A . This procedure should reduce the effect of chance variations in the value of b on the estimates of A . In the instance under consideration, this procedure gives:

$$Y = 17.8320x - 8.99 \dots \text{normal}$$

$$Y = 17.8320x + 94.15 \dots \text{lactate.}$$

The standard error of the difference between the estimates of A is then calculated. (It should be noted that A differs from the constant " a " discussed by R. A. Fisher: $A = a - b\bar{x}$, and in consequence $A_L - A_N = (a_L - a_N) - b(\bar{x}_L - \bar{x}_N)$, and $V_{(A_L - A_N)} = V_{(a_L - a_N)} + (\bar{x}_L - \bar{x}_N)^2 \cdot V_b \dots$ where V signifies the variance of the subscribed quantity.) The standard error in this instance is 29.12, and the difference in the estimates of A is 103.14. The value of t is therefore 3.542. With 21 degrees of freedom, t is 2.831 for $P = 0.01$, and therefore there is less than one chance in a hundred of observing such a difference between samples drawn from the same population. That is, the samples are drawn from different populations, and lactate does cause a significant increase in the value of A .

The five other sets of data have been examined in the same fashion, and the results of the examination are presented in the table below:

Exp.	$b_L - b_N$	s.e. of diff.	P_b	$A_L - A_N$	s.e. of diff.	P_A
D. 50.617	4.231	8.272	0.60	103.14	29.12	0.0002
D. 75.294	- 3.361	5.590	0.56	89.09	53.69	0.11
D. 100.617	- 7.856	15.928	0.65	107.73	39.08	0.015
L. 50.271	- 14.729	6.323	0.032	- 86.81	42.54	0.055
L. 75.617	- 12.534	12.927	0.34	19.15	153.36	0.90
L. 100.2000	- 2.353	8.149	0.80	15.02	90.63	0.90

Under the heading "Exp." the designation D. 50.617 means that in this experiment bird 617 has been receiving 50 mg. *per diem* of dextrorotatory lactate.

This table merits discussion in some detail. In the first place it will be seen that in every instance but one the apparent effect of lactate administration on the value of b is a reduction. However, the standard error of the estimate of the difference between the normal and lactate values of b (column three) is so large that none of these changes is significant. The general conclusion must be that there is certainly no increase in b following lactate administration, but that there may be a decrease although the errors of estimation in the present data are such that the observed decreases are not significant.

If, in point of fact, there is a decrease in b , then the estimates of the difference in A are all too low, but there is not the material to assess the magnitude of this possible effect. With the exception of the first value obtained for laevorotatory lactate the values of A are strikingly uniform within the two classes. This part of the table is self-explanatory, and it will be seen that, although in one instance the rise in A following dextrorotatory lactate does attain the level of significance, there is in this instance a very large value of the standard error. The only instance of approach to significant level in the laevorotatory series is an instance of a fall in A following lactate and need not be considered further. The last point in connection with these results is that unfortunately the standard errors of the differences in the last two instances in the laevorotatory series are very high indeed and it might be suspected that this accident was responsible for the high values of P . However, even were the standard error in either instance to be reduced to 30 the value of P would not fall below 0.54; there would still be 54 chances in 100 of observing such a difference between two samples of a uniform population having that variance.

Thus there can be very little doubt that the administration of sodium *d*-(-)lactate does not bring about an increase in either of the parameters of the regression line, whereas the administration of sodium *l*-(+)lactate brings about an increase in A but no increase in b .

That is, the regular administration of a constant dose of sodium *l*-(+)lactate tends to produce a constant increase in uric acid output, irrespective of the food

intake. Furthermore this increase represents a physiologically reasonable conversion of the lactate into uric acid. In the three experiments quoted in the table above the values of $(A_L - A_N)$ represent 55.3, 28.4 and 28.9% conversion respectively.

If the theoretical discussion earlier in this paper of the physiological significance of changes in the parameters of the regression line is valid, it follows that *l*-(+)-lactate can participate in uric acid synthesis in pigeons, whereas *d*-(-)-lactate cannot.

SUMMARY.

Sodium *d*-(-)-lactate, in daily doses containing from 50 to 100 mg. of the free acid, has no effect on the uric acid excretion of homer pigeons.

Sodium *l*-(+)-lactate, in similar amounts, increases the uric acid excretion significantly. The nature of this increase is analysed, and reasons are given for regarding it as indicative of the direct participation of *l*-(+)-lactic acid in the synthetic process.

I wish to express my thanks to Prof. Peters for his encouragement, to Prof. R. A. Fisher for his help with statistical difficulties, and to the Distillers Company's Laboratories for the gift of generous quantities of zinc ammonium lactates.

APPENDIX.

The statistical adequacy of Wiener's investigations of uric acid synthesis in hens.

Since Wiener's investigations form the cornerstone supporting the urea-conjugation hypothesis of uric acid synthesis, it is a matter of some importance if it can be shown that his results are statistically inadequate to sustain his claims.

Uric acid excretion. (g. per 24 hours.)

Bird	No lactate	Lactate	Daily dose
A	1.84		
	1.58		
	1.40	1.97	0.6 g. <i>l</i> -(+)-lactic acid
		1.50	
	1.61	1.56	
	1.61		
C	1.64		
		3.55	1.2 g. <i>dl</i> -lactic acid
		2.68	
	2.65	2.08	
	2.62		
	2.42	3.57	0.6 g. <i>l</i> -(+)-lactic acid
D		2.58	
		2.05	
		3.76	1.2 g. <i>dl</i> -lactic acid
		2.81	
	2.33	2.94	
	2.68		
E	2.35	2.76	0.6 g. <i>l</i> -(+)-lactic acid
		2.75	
		2.37	
		2.94	1.2 g. <i>dl</i> -lactic acid
		2.81	
	2.05	2.22	
	2.57		
	2.26	2.93	0.6 g. <i>l</i> -(+)-lactic acid
		3.28	
		2.03	

The daily doses are given in terms of the free acid although the acid was administered as the sodium salt.

I have chosen the results obtained with lactate, malonate and tartronate for examination. The lactate series is the most numerous and should give the least equivocal answer. The malonate and tartronate results are chosen because Wiener claimed 100 % conversion of these substances.

Wiener's method was to give hens a constant daily dose of urea and to determine the uric acid excretion in three successive 24-hour periods. The test-substance, lactate, malonate or tartronate, was then given in a constant daily dose, in addition to the urea, and the uric acid excretion in three successive 24-hour periods was again determined. The results obtained when lactate was fed are reproduced in the above table (p. 2204).

The method used for the examination of these results is that given by R. A. Fisher [1932, p. 114]. The essential steps are the determination of the variance, *i.e.* the value of $S(x - \bar{x})^2$, for each group of results, and the derivation from this of an estimate of the standard error of the difference of any two of the means. The estimate of the standard error, divided into the observed difference between the means in question, gives a value of t from which the probability of a difference of the magnitude observed occurring between samples of the same population can be estimated. If the means compared are derived respectively from series with and without lactate feeding, a high probability will signify that the series with lactate feeding belongs to the same population as the series without lactate feeding, that is, that lactate has no significant influence on uric acid excretion.

The essential results are collected in the table below:

Bird	Lactate	Diff. of means	Standard error	t
(7)A	<i>L</i> -(+)lactate	0.06	0.13	0.492
(4)C	<i>dl</i> -lactate	0.21	0.42	0.502
	<i>L</i> -(+)lactate	0.17	0.45	0.378
(4)D	<i>dl</i> -lactate	0.72	0.49	1.433
	<i>L</i> -(+)lactate	0.17	0.38	0.455
(4)E	<i>dl</i> -lactate	0.36	0.27	1.355
	<i>L</i> -(+)lactate	0.45	0.40	1.129

The figures in brackets in front of the letters designating the birds indicate the number of degrees of freedom of the data. The "Diff. of means" column shows the difference between the mean of the normal results for that bird and the mean of the results obtained during lactate feeding.

As, for 7 degrees of freedom, $t=0.543$ when $P=0.6$, and, for 4 degrees of freedom, $t=1.533$ when $P=0.2$, it follows that in the most favourable instance there is still more than one chance in five of the observed difference arising by chance between two samples from the same population.

However, it might be objected that the uniformly positive character of the change when lactate is fed must mean that there is a true rise in uric acid excretion as the result of lactate feeding, even though the rise is not significant in individual instances. This point is best tested by considering the results obtained with birds C, D and E, which should all be comparable. The average of the difference of means (see table) for *dl*-lactate for these three birds is 0.430, and the standard error of this average is 0.151. In order to test whether the average is significantly different from zero, $t=0.430/0.151$ is calculated, the answer being 2.842. As the data possess only 2 degrees of freedom, $t=2.920$ when $P=0.1$, and in consequence the average difference cannot be said to differ significantly from zero. When the results obtained with *L*-(+)-lactate are treated similarly a value 2.817 is obtained for t . That is, the variation in the observed mean rise in uric acid excretion is so great that there is at least one

chance in ten of obtaining a mean difference of zero in a series of three experiments carried out in the same manner as Wiener's.

The experimental data for malonic acid and tartronic acid are presented in the following table:

Uric acid excretion.

(g. per 24 hours.)

Bird	No addition	Malonate or tartronate	Daily dose
A	1.84		
	1.58		
	1.40	2.34	0.75 g. malonic acid
		1.72	
	1.61	1.61	
	1.61		
	1.64		
F	3.63	4.39	1.5 g. malonic acid
	3.17	3.63	
	2.56	3.16	
H	2.38	3.51	1.23 g. tartronic acid
	2.50	2.56	
	2.51	2.48	

The results of statistical examination of these results can be expressed very briefly: for A there are 7 degrees of freedom, and $t = 1.621$. When $P = 0.1$, $t = 1.895$. For F and H there are 4 degrees of freedom and the respective values of t are 1.281 and 1.160. When $P = 0.2$, $t = 1.530$. It follows that none of these differences is significant.

In conclusion, it is interesting to note the frequency with which the uric acid excretion in the first 24-hour period of lactate, malonate or tartronate administration shows a very sharp rise, which is followed in the subsequent periods by a fall which may be so great that in the third period the uric acid excreted is less than in any of the normal periods. This suggests strongly that in Wiener's work what was being observed was a washing out of urates, probably due to the diuretic effect of large amounts of the salts administered, followed by a corresponding retention of urates in the later periods. It seems very probable that the apparent increases observed would not have been obtained if the period of administration of lactate *etc.*, had been doubled.

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