NITROGENOUS EXCRETION BY EMBRYOS OF THE VIVIPAROUS SNAKE *THAMNOPHIS S. SIRTALIS* (L.)

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(*Received* 18 *December* 1955)

Functional considerations of the placenta of saurian reptiles are limited virtually to assumptions based on known embryonic requirements and structural placental features (Weekes, 1935). It appeared that the nature of embryonic excretion might profitably be investigated in the garter snake whose young are born alive. The extreme solubility of urea, and the fact that the black snake embryo excretes only 20% of its nitrogenous waste as uric acid (Clark, 1953), suggest the possibility that embryos of a placental saurian reptile might pass urea and ammonium salts to the blood stream of the mother; whether some of the soluble nitrogenous excreta are eliminated through the placenta or whether all nitrogenous excreta are retained in the foetus and adnexa, the degree to which the embryo is dependent upon uric acid is of interest. Accordingly, assays of urea, ammonia and uric acid in the developing embryo were undertaken.

MATERIALS AND METHODS

Urea and ammonia were measured by the technique of Conway (1947), and uric acid was determined colorimetrically according to the method of Brown (1945). Periodically, blood samples were drawn from the mother by severing the tail, and comparisons of urea and ammonia were made with non-pregnant females and males. The measurements of ammonia and urea production by the adult snake were obtained by confining the snake in a glass container, clearing the cloaca by pressure, and washing the snake with 100 ml. water which served as diluent for the excreta.

Embryonic tissues were obtained by serial removal from the mother after anaesthetization with Nembutal (Clark, 1937). The tissues for analysis were weighed on a Roller-Smith Balance and a quantity of water was added which would bring the urea or uric acid to a concentration suitable for accurate determination by the techniques employed. The tissues were then homogenized in a Potter homogenizer, and aliquots were taken for assay of ammonia, urea and uric acid.

Dry weights were obtained by treatment in a vacuum oven at 30° C. at approximately 50 mm. pressure to constant weight. Protein assays were made according

* This investigation was supported in part by a research grant (G–3827) from the National Institutes of Health, U.S. Public Health Service.

to the method of Willits, Coe & Ogg (1949). Ashing was accomplished by preliminary heating in a muffle furnace for 72 hr. at 500° C., and by direct heating in a porcelain crucible over a gas flame (*ca.* 1000° C.) to constant weight.

Twelve female garter snakes provided embryos for the analyses reported below. They were collected locally during May and June. While in captivity they were fed earthworms and were kept at ordinary laboratory temperatures. The procedure for determining embryonic age is described elsewhere (Clark, Florio & Hurowitz, 1955).

The determination of transfer of ammonia from urea to uric acid was accomplished by injection of a known quantity of ^{15}N urea and subsequent recovery of ^{15}N from excreted uric acid. Injections of 1·5 ml. of 100 mg. % ^{15}N urea (15 % excess of ^{15}N) were made intraperitoneally in five males and five pregnant females and into the yolk sacs of embryos (0·75 ml. into each of two) of five pregnant females. Excreta were collected at 24 hr. intervals by washing cloacal contents into a vial. The excreta were freed of ammonia and urea by repeated washing and centrifugation; uric acid of the residue was dissolved in 0·25 % Li_2CO_3. The dissolved uric acid was degraded by the method of Edson & Krebs (1937), consisting of oxidation by MnO_2 at 40° C., alkaline hydrolysis and acid hydrolysis in a steam bath, enzymic hydrolysis of urea, collection of ammonia in 0·05 HCl, and oxidation of NH_4Cl to N_2 gas *in vacuo* (Rittenberg, 1947). Gas samples were analysed spectrometrically for $^{29}N/^{28}N$ ratio, which was compared with the ratio obtained for gas similarly derived from chemically pure uric acid. (The authors are indebted to Dr S. Friedland and Mr George Strakna for co-operation in making the analyses.)

RESULTS

Growth. Nitrogen metabolism during embryonic development is of necessity a composite of activities related to growth, differentiation and maintenance. To provide a frame of reference for the data on excretion and to evaluate the role of the placenta in nutrition and excretion, some aspects of protoplasmic increase will be considered.

The curve of wet-weight increase of the embryo is presented in Fig. 1*a*. Relationship of wet weight to dry weight is described in Table 1. These data confirm the common observation that early in development, wet weight increases rapidly, and in later stages, more slowly, whereas the reverse is true for dry weight. Rate of increase for embryonic dry weight in terms of k values is stated in Table 2.

Because the garter snake embryo develops from a highly telolecithal egg, but establishes a placental union with the mother, distinction between the ovarian and placental roles in nutrition is difficult. It was pointed out (Clark *et al.* 1955) that water, and probably amino-acids, pass the placenta. Some evidence with regard to protein exchange is provided by comparison of total initial protein with the amount found in neonates (Table 1).

Wet and dry weights of yolk and yolk sac were obtained over the entire incubation period; ratio of water and dry substance remained constant throughout the

Table 1. *Dry weight in relation to wet weight in garter snake development*

Age (days)	Embryo (mg.)				Yolk and yolk sac (mg.)			Total. Embryo and adnexa (mg.)			
	Wet	Dry	Protein	Ash	Wet	Dry	Protein	Wet	Dry	Protein	Ash
0	—	—	—	—	490	255	132	490	255	132	—
0	—	—	—	—	453	221	111	453	221	111	—
3	—	—	—	—	—	—	—	462	228	—	17·1
3	—	—	—	—	—	—	—	680	294	—	24·1
7	—	—	—	—	—	—	—	469	221	131	—
7	—	—	—	—	—	—	—	659	285	117	—
21	—	—	—	—	—	—	—	665	198	79	—
21	—	—	—	—	—	—	—	453	235	117	—
22	158	12	—	—	601	262	107	759	274	—	—
25	219	18	9	—	735	325	150	954	343	—	—
25	220	18	11	—	620	322	—	840	341	—	—
26	249	21	11	—	731	329	144	980	350	—	—
35	307	24	15	—	691	299	136	998	323	151	—
35	242	—	—	—	708	314	88	1006	311	122	—
35	—	—	—	—	687	159	45	1305	350	135	—
38	366	28	15	—	729	338	150	1095	366	165	—
49	562	59	29	—	647	312	123	1209	371	152	—
50	581	50	30	—	633	297	78	1214	347	108	—
50	566	52	—	4·6	614	281	—	1180	333	—	—
51	625	43	31	—	584	316	—	1209	359	—	—
57	758	64	41	—	461	210	—	1219	274	—	—
57	768	72	—	10·2	232	106	—	1000	178	—	—
59	824	96	53	—	531	237	131	1355	333	184	—
59	820	66	—	—	455	208	—	1275	274	—	—
59	816	64	—	7·8	459	249	—	1275	313	—	—
67	1098	129	81	—	213	97	—	1311	226	—	—
67	1004	115	—	16·1	243	111	—	1247	226	—	—
70	1246	189	91	—	328	151	87	1574	340	178	—
70	1244	223	—	24·8	0	0	0	1244	223	—	—
70	1219	247	147	—	0	0	0	1219	247	147	—
70	1287	244	—	26·8	0	0	0	1287	244	—	—
70	1242	240	153	—	0	0	0	1242	240	153	—
70	1303	231	143	—	0	0	0	1303	231	143	—

Table 2. *k values for growth rate and rates of accumulation of excreted nitrogen*

(From the equation $W = Ae^{kt}$, where W = weight, A = ln W when $T = 0$, e = base of natural logarithms, T = time (age). k value with period covered (days) shown in parentheses.)

Wet weight	0·197 (0–25) 0·055 (25–53) 0·010 (55–72)
Dry weight	0·047 (20–57) 0·094 (57–71)
Total N	0·11 (0–33) 0·028 (33–72)
Ammonia N	0·050 (0–72)
Urea N	0·092 (0–32) 0·032 (33–72)
Uric acid N	0·073 (0–72)

gestation period (water = 54·3 ± 3·0%). The dry weight of the yolk and yolk sac rises to a peak on the 25th day, which is maintained until the 50th day, after which the dry substance becomes depleted with resorption of the yolk sac. Intrauterine dependence on stored yolk by garter snake embryos is in contrast with the sparing of yolk for post-partum nutrition of *Vipera berus* (Bellairs, Griffiths & Bellairs, 1955).

Table 3. *Excreta recovered from total garter snake egg in mg. nitrogen*

Age (days)	Embryo weight (mg.)	NH_3	Urea	Uric acid	Total
Unfert.	—		0·047	—	0·047 est.
6	—		0·021	—	0·021 est.
17	—		0·029	—	0·029 est.
18	45		0·045	—	0·045 est.
22	100	0·044	0·161	0·006	0·211
33	360	0·037	0·529	0·016	0·582
34	269	0·035	0·290	0·010	0·335
35	273	0·074	0·452	0·016	0·542
38	401		0·492	—	0·522 est.
41	373	0·093	0·409	0·024	0·526
43	434	0·094	0·374	0·035	0·503
43	459	0·120	0·527	0·031	0·678
45	533	0·069	0·410	0·016	0·495
49	565	0·052	0·517	0·018	0·587
50	636	0·120	0·513	0·039	0·672
50	907	0·133	—	—	—
60	901	0·094	0·643	0·074	0·811
60	984	0·080	0·690	0·087	0·857
61	904	0·238	0·775	0·064	1·077
61	1308	0·276	0·725	0·100	1·101
63	1193	0·089	0·880	0·036	1·005
65	929	0·084	0·825	0·076	0·985
65	1446	0·124	0·957	0·098	1·179
67	686	0·201	0·775	0·027	1·003
69	1500	0·095	0·867	0·110	1·072
69	1510	0·117	1·030	0·117	1·264
70	994	0·111	0·770	0·104	0·985
72	1000	0·139	0·582	0·120	0·841
72	1406	0·390	0·733	0·390	1·513

Protein analyses (Table 1) suggest that the principal dry substance lost during the early decline is protein; since the protein content of the neonate is greater (approximately 30 mg.) than that originally present, it is apparent that this amount was supplied through the placenta, presumably as amino-acids. Later, it will be shown that the excreta per embryo would require the degradation of approximately 16 mg. protein; hence, a minimum of 45 mg. per embryo will have passed the placenta during the incubation period.

There is apparently a slight increase in ash content of the embryo in comparison with the original store available, both in absolute quantity and in percentage composition (Table 1). This would imply that a small portion of the ash of the embryo is derived by transfer across the placenta; the implication is not construed as denial of a more extensive transplacental traffic of inorganic substances in both directions.

Excretion. The excretory data are recorded in Table 3. Graphic representations of these data in Figs. 1 *a* and 1 *b* express more clearly interrelationships and trends. Total excreted nitrogen (Fig. 1 *a*) follows the pattern of growth, though it will be seen (Table 2) that the rate of accumulation of waste products in the early period of development ($k=0{\cdot}11$) is less than the rate of growth of wet weight ($k=0{\cdot}197$) and greater than that of dry weight ($k=0{\cdot}047$). This would point to a relatively

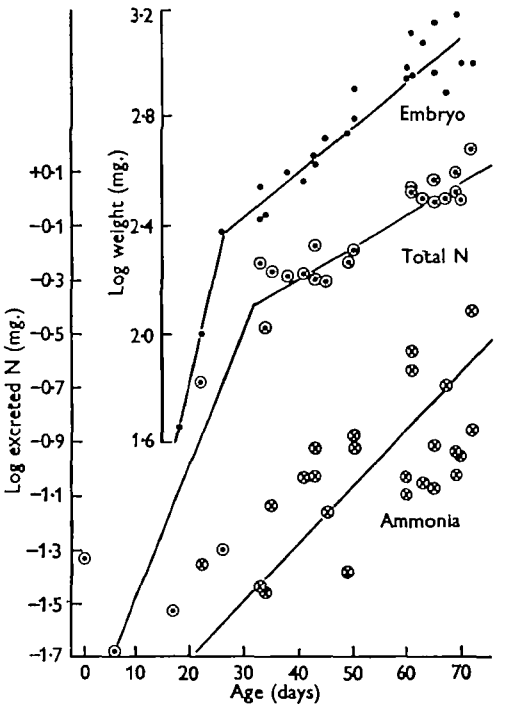

Fig. 1 *a*

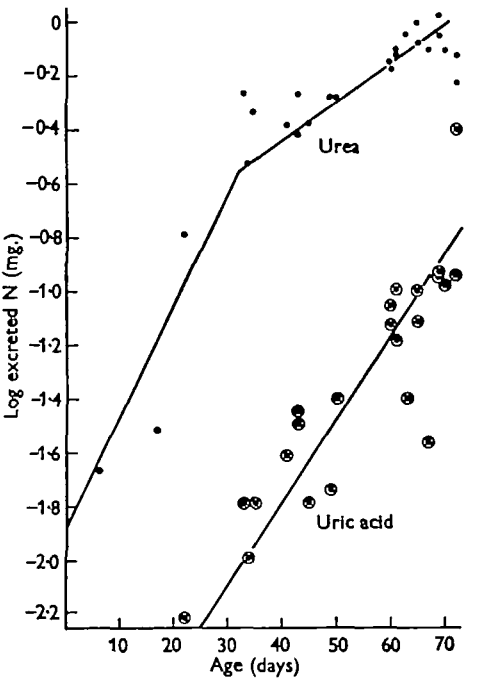

Fig. 2 *b*

Fig. 1 *a*. Cumulative excreta of the garter snake embryo in relation to growth, semilog plot. Ordinate: upper curve, log wet weight; middle curve, log accumulated excreted N of all types; lower curve, log accumulated ammonia N. Abscissa: age in days. Equations for growth curve: $y=0{\cdot}0945x-0{\cdot}04$ (0–25 days); $y=0{\cdot}022x+1{\cdot}72$ (25–53 days); $y=0{\cdot}0044x+2{\cdot}676$ (55–72 days). Equations for total excreted N are: $y=0{\cdot}048x+8{\cdot}06$ (0–33 days); $y=0{\cdot}012x+9{\cdot}22$ (33–72 days). Equation for ammonia N is: $y=0{\cdot}0215x+7{\cdot}86$ (0–72 days).

Fig. 1 *b*. Semilog plot of accumulated urea N and uric acid N. Ordinate and abscissa as in Fig. 1 *a*. Equations for urea are: $y=0{\cdot}040x+8{\cdot}24$ (0–32 days); $y=0{\cdot}013x+9{\cdot}06$ (33–72 days). Equation for uric acid N is : $y=0{\cdot}0319x+6{\cdot}91$ (0–72 days).

greater decomposition of protein early in development. When considered on the basis of unit weight, this observation is more apparent. In Fig. 2 it is seen that the rate of excretion per gram of tissue early in development is almost three times the rate at hatching. It would appear, therefore, that protein is being used as an energy source to a greater extent in the early period of development. (Support for this view comes also from study of other reptiles and the chick, in which the relationship described is repeated.) These data alone do not deny the hypothesis

of Needham (1931) that the principal energy sources of development are successively carbohydrate, protein and fat. It would seem, however, that protein should be given serious consideration as a primary energy source in early development.

Ammonia accumulates at a constant rate ($k = 0{\cdot}055$); since the rate of accumulation of embryonic wet-weight decreases, it follows that the concentration of ammonia with respect to the embryo increases. Ammonia may be disposed of in

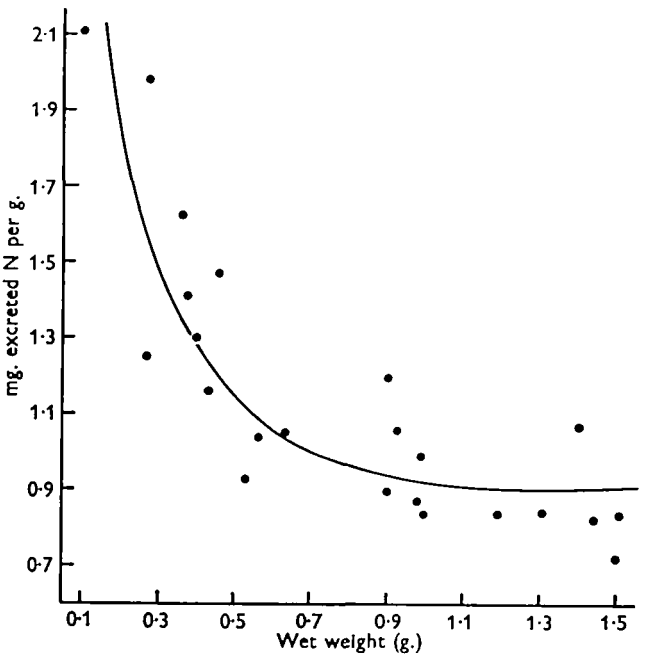

Fig. 2. Rate of excretion of nitrogen in relation to age in the garter snake embryo.

several ways: (1) urea synthesis, (2) uric acid synthesis, (3) transamination, or (4) transplacental migration. Since the concentration in embryonic tissues (and the egg as a whole) increases, it would appear that these methods are inadequate to dispose of the amount of ammonia produced; however, the increase in concentration is obviously not deleterious to the developmental processes.

The rate of uric acid accumulation is also constant; initially very small amounts are detectable, and the rate of accumulation is relatively high ($k = 0{\cdot}073$). Uric acid accumulation is apparently contingent upon development of the allantois, since it occurs principally after 3 weeks of development.

Urea accumulates rapidly during the first month ($k = 0{\cdot}092$), but after this time at a more moderate rate ($k = 0{\cdot}032$). On the basis of nutritional data above, the placenta is presumably functional after about 20 days of development. Since urea is extremely soluble, it is reasonable to suppose that it is lost through the placenta.

Since uric acid accumulation occurs principally after the period of rapid urea accumulation, it is possible that the decline in rate of urea accumulation may, in part, be the result of intra-embryonic conversion to uric acid. A similar interpretation would appear to be appropriate for data obtained by injection of ^{15}N urea (see below).

Table 4. *Comparison of excreta in garter snake and black snake embryos (mg.)*

	Weight (g.) at hatching of birth	Total excreted nitrogen	Total excreted nitrogen per gram	Percentage of total excreted nitrogen		
				Ammonia	Urea	Uric acid
Black snake	7·0	12·55	1·80	20·0	60·0	20·0
Garter snake (recovered)	1·4	1·41	1·00	23·4	60·3	16·3
Garter snake (calculated)	1·4	2·52	1·80	(90·9)		9·1

Regardless of the explanation for the mechanism of decline in urea accumulation, the importance of urea in the excretory pattern of the embryonic garter snake is attested by the similar decline in total nitrogenous waste.

Comparison of the excretory data of the garter snake and the black snake is made in Table 4. The partition of the recovered nitrogenous end products in the two species corresponds quite closely. Excretion per gram is far less in the garter snake (56%). Presuming the same composition of the two neonatal snakes that have attained similar physiological and morphological maturity, it would appear that some of the garter snake excreta are missing. To establish the same excretory rate in the garter snake would require the excretion of 2·52 mg. nitrogen. It would therefore appear that 1·11 mg. per embryo was lost through the placenta; since it is not possible to measure quantitatively the amounts of ammonia and urea which pass the placenta, the two have been combined in Table 4. It will be noted that the relative importance of uric acid in the placental species is reduced. Evidence pertaining to the role of the placenta in embryonic excretion is derived from three sets of data; they are described below.

Transplacental passage of urea

(1) *Blood urea.* A mother carrying fifteen embryos would be expected to receive a minimum of 36 mg. urea during the period of gestation, on the basis of the calculations above. Assuming that all were released during the latter half of the gestation period, the average rate of passage would be of the order of 0·05 mg. urea/hr.—sufficient to cause an increase of approximately 1 mg.% in maternal blood content. Accordingly, urea assays of maternal blood were made throughout gestation, for which males and non-pregnant females served as controls. Average values for males throughout the observation period was 4·24 mg.% (2·95–6·15), and for non-pregnant females, 5·01 mg.% (2·95–6·05); pregnant females during

the first half of the gestation period averaged 5·13 mg.% (3·50–7·40) and during the latter half, 6·93 mg.% (2·50–20·0). These data are suggestive rather than conclusive, but indicate transplacental loss of urea. They support the calculations above, but, because of variability and the demonstrated maternal urease activity (see below), cannot be said to verify them.

(2) *Excreted urea plus ammonia.* It was thought that if urea and ammonia passed through the placenta in the quantities calculated above, and were eliminated without change through the maternal kidneys, they could be recovered in the cloaca. Therefore, towards the end of gestation, two pregnant females and a non-pregnant female, all closely similar in size, were isolated in glass containers. Their combined urea and ammonia output was measured daily over an 8-day period. Average daily output of the two pregnant females was 2·0 mg.; that of the non-pregnant female was 2·2 mg. A third pregnant female was given three injections of 0·8 ml. 500 mg.% urea into the body cavity on alternate days (a total of 12·0 mg. urea). Her average output was 1·9 mg. per day.

Assays of liver and kidney of adults for urease were made, and both showed extensive activity. It was therefore suspected that urea, either artificially supplied or derived from the embryos, is converted to carbon dioxide and ammonia, and the latter may be incorporated into the uric acid molecule.

(3) *Conversion of* ^{15}N *urea.* In order to determine whether urease, shown to be active *in vitro*, was also physiologically active, the procedure outlined above (methods) was carried out. The results of the analysis are shown in Table 5.

Table 5. *Conversion of urea to uric acid (garter snake) after 48 hr.*

	^{15}N injected as urea (converted to ml. gas)	^{15}N recovered from uric acid of excreta (ml. gas)	Atom percentage excess ^{15}N recovered	Percentage ^{15}N injected recovered
Males (intraperitoneal)	0·078	0·045	3·32	57·0
Females (intraperitoneal)	0·078	0·025	1·52	32·0
Females (yolk sac)	0·078	0·017	0·75	22·0

The data point conclusively to a conversion of injected urea to uric acid. Since a quantitative statement would entail collection of 100% excreted uric acid, and preservation of this through the various steps of the process, and measurement of residual ^{15}N in the injected animal, the foregoing data must be regarded as estimates. The estimate of percentage recovery is, however, a conservative one.

It is believed that the injected urea is hydrolysed by urease, and that the resultant ammonia is incorporated into the uric acid molecule. Such a hypothesis explains the data of Table 5, the failure of injected urea ((2) above) to appear as urea, and the relatively slight increase in blood urea of pregnant females. It is not proposed that all of the embryonic urea which escapes into the maternal blood stream is subjected to urease hydrolysis, since a large amount of urea is excreted by the garter snake. The lower percentage conversion of intra-embryonically

injected urea to adult uric acid may point to the operation of the same mechanism within the embryonic confines. Because of the small quantities of uric acid available from an embryo, this problem could not be pursued. However, the fact that a significant percentage of the injected urea was recovered from the maternal excreta demonstrates conclusively the placental role in embryonic excretion.

DISCUSSION

Four aspects of the present data would seem to be of interest: (1) relation of the placenta to embryonic excretion in a reptile, (2) similarity of the mechanism of excretion in this, a viviparous species, to that of the black snake, an ovoviviparous species, (3) the identification of urease in another uric-acid producing animal, and (4) the indication that protein, particularly in early development, may be an important energy source.

The morphological findings of Weekes (1935) with respect to the reptilian placenta invite speculation, but the present data are believed to be the first which demonstrate a placental role in excretion. The concentrations of embryonic urea during the course of gestation are not so high that transplacental passage of urea may be regarded as a physiological or evolutionary requirement. It would rather appear that the extreme solubility of urea makes it inevitable that some must escape from the embryonic confines. It could hardly be argued that the placenta has survival value on account of excretory advantages alone.

No evidence has come to light which would indicate a qualitative difference in the basic mechanisms of excretion between the garter snake, a non-cleidoic, and the black snake, a cleidoic form. Since the possible methods of eliminating nitrogen are so limited, a reduction of the relative importance of uric acid is the only change which might reasonably be expected.

If our estimate of the urea passing through the placenta is approximately correct, the placenta cannot be regarded as constituting a barrier to the elimination of urea. The garter snake, then, is an exception to Needham's (1950) theory that the excretory mechanism of the adult vertebrate is that which is imposed upon it by conditions of embryonic development.

The identification of urease in the adult garter snake and the presence of urease throughout the development of the chick (Clark, Fischer & Florio, 1953) suggests that the synthesis of uric acid may be a means of getting rid of the ammonia which is produced by the breakdown of urea. Urease is found in other animals which produce uric acid, namely, in the chick at 4 weeks post-hatching, in various tissues of the mouse and rabbit, and has been shown to be present as well in the rat, cat, dog, mouse and frog (Fitzgerald, 1948) and in *Helix* (Baldwin & Needham, 1935). Since it has been found abundantly in the liver and kidney of the newt and frog (in which its activity would create no serious problem because ammonium salts might readily be eliminated to an aqueous environment or enter into other metabolic pathways), the possibility exists that it arose in the amphibia and has been transmitted genetically to eptilian, avian and mammalian descendants. The

uric acid mechanism in terrestrial animals would then be construed as an evolutionary essential, whose fundamental inception was occasioned by necessity of getting rid of ammonia rather than purines. A functional relationship between urease activity and uric acid synthesis seems sufficiently well established to warrant further pursuit of the problem.

The fact is established that the rate of excretion early in garter snake development is three times greater per unit mass of tissue than that at the end of development; since similar data are available for other vertebrates (unpublished), it would appear that a painstaking analysis of energy sources in both the chick and reptiles might be fruitful.

SUMMARY

1. The garter snake embryo excretes an estimated 2·52 mg. nitrogen, of which 1·4 mg. is recoverable from the embryonic confines. The recovered excreta consist of 16·3% uric acid, 23·4% ammonia and 60·3% urea.

2. The placenta is believed, therefore, to transmit to the mother 1·11 mg. nitrogen per embryo, and it is estimated that it transmits to the embryo approximately 45 mg. of protein as amino-acids.

3. Evidence is presented which suggests that protein may be a principal source of energy, particularly early in development.

4. Growth in terms of wet weight, dry weight, and protein is described.

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