if the fish drinks, but nothing is known in regard to "chloride cells" or a possible salt-secreting function of the nodular gland associated with the cloacal pouch (*17*).

Some information is available regarding the composition of the aqueous and vitreous humors in the eye of teleosts, but Cl is stated to be lower in both chambers than in the blood (*18*). More is known regarding the ocular fluids of elasmobranchs: the total osmolality of the aqueous humor is slightly below that of the blood; the major inorganic ions and urea follow the same pattern (*12, 19*). Our data on urea are compatible with these results. If comparison is made with heart blood, the aqueous humor appears to be hypertonic, as is true of elasmobranch vitreous humor (*19*).

The work reported above was essentially completed (*20*) independently of that of Brown and Brown (*7*), and our mutual findings are complementary. They found urea concentrations of the order of 280 mmole per kilogram of liver (wet weight), and we report an average of 355 mmole per liter of serum. Clearly the tissues are in approximate equilibrium with the blood.

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References and Notes

1. H. W. Smith, *From Fish to Philosopher* (Little Brown, Boston, 1953; Ciba Pharmaceutical Products Company, Summit, N.J., revised edition, 1959). Another of Smith's predictions appears to be confirmed by Millot and Anthony, *Compt. Rend.* **251**, 442 (1960). Smith postulated that if *Latimeria* is ureotelic, like an elasmobranch, it should have a cleidoic egg, and this indeed appears to be the case. Nonencapsulated ova (70 mm in diameter) were found in the oviduct of a mature female.
2. K. S. Thomson, *Science* **153**, 999 (1966). We are indebted to Dr. Thomson for the privilege of taking blood samples and for additional information on the probable depth at which capture occurred. The specimen was taken at night with a handline baited with small *Cypselurus bahiensis*, at a depth of 150 to 200 meters, off Iconi, Grand Comore.
3. S. de Prailauné, *Compt. Rend. Soc. Biol.* **149**, 655 (1955).
4. A. B. Beck, *Australian J. Zool.* 4, 1 (1956).
5. These and other ultramicromethods were developed in connection with studies of the blood of *Fundulus heteroclitus*; reserved for publication at a later date. Most of the determinations were made on 5-μl samples but serum osmolality required 10 μl, and HCO_3 required 20 or 30 μl; 1- or 2-μl samples sufficed for determinations of urea and nonprotein nitrogen; the electrophoretic runs required 0.3 μl.
6. J. F. Fekete and N. Kerenyi, *Can. Med. Ass. J.* **92**, 970 (1965).
7. G. W. Brown and S. G. Brown, *Science*, this issue. We thank Dr. Brown for the privilege of reading the aforesaid manuscript and for encouragement in the preparation of our own.
8. N. H. Grant and H. E. Alburn, *Nature* **212**, 194 (1966).
9. We thank Dr. M. S. Gordon for his data on salinity and temperature at two stations in the Anjouan area, close to the nearby island of Grand Comore.
10. H. W. Smith, *J. Biol. Chem.* **81**, 71, 407 (1929); J. D. Robertson, *J. Exp. Biol.* **31**, 424 (1953); E. L. Becker, R. Bird, J. W. Kelly, J. Schilling, S. Solomon, N. Young, *Physiol. Zool.* **31**, 224 (1958); M. R. Urist, *Endocrinology* **69**, 778 (1961); R. Fänge and K. Fugelli, *Sarsia* **10**, 27 (1963); P. S. Enger, *Comp. Biochem. Physiol.* **11**, 131 (1964); G. R. Bernard, R. A. Wynn, G. G. Wynn, *Biol. Bull.* **130**, 18 (1966); J. B. Hunn and P. F. Robinson, *Chesapeake Sci.* **7**, 173 (1966).
11. W. S. Platner, *Amer. J. Physiol.* **161**, 399 (1950).
12. R. F. Doolittle, C. Thomas, W. Stone, *Science* **132**, 36 (1960).
13. L. Brull and Y. Cuypers, *J. Mar. Biol. Ass. U.K.* **34**, 637 (1955).
14. J. Hodler, H. O. Heineman, A. P. Fishman, H. W. Smith, *Amer. J. Physiol.* **183**, 155 (1955); Q. C. Haning and A. M. Thompson, *Comp. Biochem. Physiol.* **15**, 17 (1965); E. C. Black, G. T. Manning, K. Hayashi, *J. Fish. Res. Board Can.* **23**, 783 (1966).
15. H. W. Smith, *J. Biol. Chem.* **88**, 97 (1930); P. A. Jannsens and P. P. Cohen, *Science* **152**, 358 (1966); R. P. Forster and L. Goldstein, *ibid.* **153**, 1650 (1966); G. W. Brown, Jr., J. James, R. J. Henderson, W. N. Thomas, R. O. Robinson, A. L. Thompson, E. Brown, S. G. Brown, *ibid.* **153**, 1653 (1966); M. Gilles-Baillien and E. Schoffeniels, *Ann. Soc. Roy. Zool. Belg.* **95**, 77 (1966); L. McClanahan, *Comp. Biochem. Physiol.*, in press.
16. M. S. Gordon, K. Schmidt-Nielsen, H. M. Kelly, *J. Exp. Biol.* **38**, 659 (1961); K. Schmidt-Nielsen and P. Lee, *ibid.* **39**, 167 (1962); M. S. Gordon and V. A. Tucker, *ibid.* **42**, 437 (1965).
17. J. Millot and J. Anthony, in *Traité de Zoologie*, P. P. Grassé, Ed. (Masson, Paris, 1958), vol. 13 (3), pp. 2553–597; *Bull. Mus. Nat. Hist. Natur.* **32** 287 (1960).
18. J. R. Hoffert and P. O. Fromm, *Comp. Biochem. Physiol.* **18**, 333 (1966); Y. Derrien, *Ann. Inst. Oceanogr.* **20**, 115 (1940).
19. W. Stone and W. C. Dewel, *Biol. Bull.* **123**, 513 (1962), in disagreement with T. H. Maren, *Comp. Biochem. Physiol.* **5**, 193 (1962).
20. F. B. Grant and G. E. Pickford, *Amer. Zool.* (abstr.) **6**, 345 (1966).
21. Supported by NSF grant GB-0132.

12 December 1966

Urea and Its Formation in Coelacanth Liver

Abstract. *Urea occurs in liver of the coelacanth* Latimeria chalumnae *to the extent of about 1.7 percent by weight. It was determined quantitatively by reaction with 1-phenyl-1,2-propanedione-2-oxime (Archibald reagent) and by measurement of ammonia released upon treatment with urease. Arginase and ornithine carbamoyltransferase, enzymes instrumental in the formation of urea in typical ureotelic vertebrates, occur in homogenates of coelacanth liver. Formed in part by the ornithine-urea cycle, urea may have an osmoregulatory function in the coelacanth as it has in elasmobranchs.*

Anatomical studies (*1*) suggest a close relation between the coelacanth *Latimeria chalumnae* and the fossil rhipidistian fishes. The latter are supposed to be closely related to the antecedants of Amphibia and subsequently emerging higher vertebrates (*2*). Our biochemical studies on liver of the coelacanth

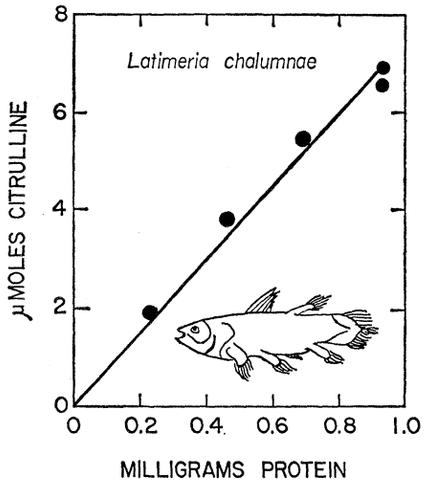

Fig. 1. Ornithine carbamoyltransferase of coelacanth liver: protein concentration study. Conditions as given in Table 3 but with varying amounts of protein (30 minutes).

(*3*) suggest that *Latimeria* has an intermediary nitrogen metabolism more closely akin to that of the Elasmobranchii (sharks, rays, and skates), Dipnoi (lungfish), and Amphibia than to that of the Teleostei (higher bony fishes).

Elasmobranchs, lungfish (*Protopterus*), and amphibians have the functional ornithine-urea cycle described by Krebs and Henseleit (*4*). Hence members of these three classes of vertebrates can synthesize enzymically, *de novo*, urea from carbon dioxide, ammonia, and amino groups of amino acids. While elasmobranchs maintain high concentrations of urea in tissues and plasma (1 to 3 percent by weight) (*5*), the estivating lungfish *Protopterus aethiopicus* (*6*) and the marine frog *Rana cancrivora* (*7*) accumulate correspondingly large amounts of urea in tissues and in the blood. The osmotic importance of elasmobranch blood urea is well recognized (*5*). On the other hand, teleostean fishes have been supposed to lack the functional ornithine-urea cycle (*8*), and there is no evidence to suggest that they ever build up appreciable quantities of urea in tissues or blood (*5*).

We have found that (Table 1) coelacanth liver contains approximately 17 mg of urea per gram of liver (wet weight) (1.7 percent), a value comparable to that of the livers of many sharks (*5*). The quantity of urea per gram of liver (Table 1) was estimated on 10-percent water homogenates (*9*) of coelacanth liver in two ways: (i) by reaction with 1-phenyl-1,2-propanedione-2-oxime (*10*), and (ii) by the amount

of ammonia released (nesslerization) upon treatment with urease (*11, 12*).

Small portions of the homogenate (spotted directly and after removal of protein by heat coagulation) were chromatographed with a mixture of *n*-butanol, acetic acid, and water (4 : 1 : 5, upper phase) on Whatman No. 1 filter paper followed by buffered phenol in a second direction (*13*). The developed chromatograms were sprayed with *p*-dimethylaminobenzaldehyde (2 percent in alcoholic 10 percent HCl). Intense yellow spots having the same R_F values as authentic urea (0.51 and 0.80, respectively) were obtained for the homogenate. Urea was the only component on the chromatograms visible from 10 μl of homogenate after reaction with *p*-dimethylaminobenzaldehyde. At least eight ninhydrin reactive spots were found on the chromatograms.

Allantoin, which yields essentially the same spectrum with 1-phenyl-1,2-propanedione-2-oxime as does urea (E_{max}, 540 to 545 mμ), was ruled out as being the chromogenic material reacting with the substituted oxime by the two-way chromatography and by the stoichiometric recovery (within experimental error) of calculated amounts of ammonia by urease treatment. Independent proof that the material was not allantoin was necessary since the urease preparation used contained some residual allantoinase (*11*) and possibly allantoicase (*11*) activity; thus allantoin in the sample could have been a potential source of the ammonia measured.

Paper chromatograms spotted with homogenate failed to yield any trace of glyoxylic acid when they were sprayed with 2,4-dinitrophenylhydrazine in HCl, followed by base. Hydrolysis of allantoin and allantoic acid to urea would yield glyoxylic acid; thus allantoin and allantoic acid appear to be ruled out as contributing significantly to the high concentration of urea found in coelacanth liver.

In elasmobranchs (*14*), lungfish (*15*), and Amphibia (*16*), urea may arise degradatively from purines, biosynthetically from the ornithine-urea cycle (*8, 17*), or from dietary arginine, or from any combination of these. Production of urea from the last two sources would require the presence of the enzyme arginase (*11*). We have demonstrated conclusively the enzymic formation of urea from L-arginine with a homogenate of coelacanth liver (Table 2). In the assay for arginase (*18*), little, if any, urea was formed if L-arginine was omitted from the reaction mixture or if the homogenate was boiled. Omission of $MnCl_2$ decreased the conversion of L-arginine to urea, and manganous ion is known to be an activator of arginase. The material produced in the assay was shown to be urea by the absence of the accumulation of product reacting with 1-phenyl-1,2-propanedione-2-oxime in the incubation tube containing urease. Optical specificity of the coelacanth arginase is for L-arginine, and D-arginine in the assay system did not lead to urea production.

Table 1. Urea content of coelacanth liver. A portion (0.30 ml) of a 10 percent homogenate (held at 70°C for 10 minutes and centrifuged) was incubated for 30 minutes at 38°C in the presence of 600 μmole of potassium phosphate buffer, *p*H 7.0, and 1.0 mg of urease (Sigma Type V); final volume, 1.00 ml. Ammonia and urea standards were incubated as indicated. Reactions were stopped with 4.00 ml of 0.50*M* $HClO_4$, the tubes were centrifuged, and ammonia was determined on portions by nesslerization. Urea was determined by the Archibald (*10*) procedure with 1-phenyl-1,2-propanedione-2-oxime. Determinations in triplicate; mean value and mean deviation indicated for replicates.

Amount incubated		Amount of ammonia recovered	
With urease (μmole)	Without urease (μmole)	With urease (μmole)*	Without urease (μmole)
	Ammonia standards		
	18.0		17.4 ± 0.1 (theory, 18.0)
	Urea standards		
9.00		17.9 ± 1.1 (theory, 18.0)	
	Urea of homogenate		
8.80 ± 0.20†	8.80 ± 0.20†	16.1 ± 1.1‡ (theory, 17.6)	0.53 ± 0.25 (theory, none)

*Corrected for urease blank. †As estimated by the Archibald (*10*) procedure. ‡Corresponds to 268±18 μmole of urea per gram of liver (wet weight).

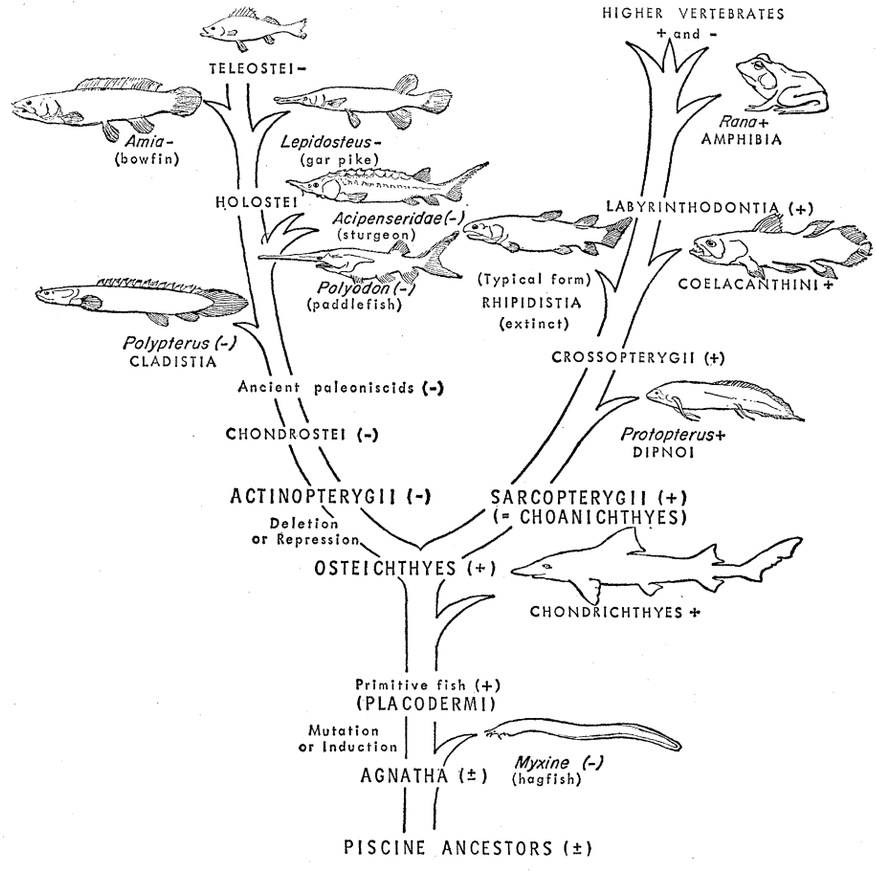

Fig. 2. Evolutionary tree of the vertebrates and occurrence of the ornithine-urea cycle. Format after the style of Romer (*23*). Presence of cycle, +; absence, —; presumed presence, (+); presumed absence, (—). Assignments based on the numerous studies cited in text.

Table 2. Arginase activity of coelacanth liver homogenate. The complete system contained 85 μmole of L-arginine, *p*H 9.7; 60 μmole of glycine (Na) buffer, *p*H 9.5; 0.050 μmole of $MnCl_2$; volume equivalent to 4.0 μl of a 10-percent water homogenate of liver and containing 48 μg protein; water to a final volume of 1.00 ml; incubation at approximately 26°C for intervals indicated. Reactions were stopped with 2.50 ml 0.50*M* $HClO_4$; urea was estimated by the Archibald procedure (*10*) on small portions with 1-phenyl-1,2-propanedione-2-oxime; absorbance was read in rectangular cuvets in the DU spectrophotometer (with a path length of 1.00 cm). Urease (1 mg) (Sigma Type V) was added where indicated; D-arginine was substituted for L-arginine (same molar concentration) where indicated. A standard curve was prepared from readings of urea standards incubated in the complete system less homogenate.

Alterations to system	Incubation (min)	Absorbance* (540 mμ)	Urea found (μmole)
None	30	0.079	0.136
None	60	.184	.316
None	120	.410	.706
Less L-arginine	60	.035	.060
Less $MnCl_2$	60	.081	.140
Plus ureases	60	−0.023†	
Boiled homogenate	60	.011	.020
D-Arginine substituted for L-arginine	60	.010	.017

* Corrected for the zero-time control (absorbance, 0.043 read against system containing no homogenate). † Negative value reflects the removal of urea produced in the assay as well as removal of endogenous urea.

We obtained an arginase activity of 13.8 ± 0.3 μmole of urea per minute per gram of liver. The activity in fresh liver is probably much higher. The assays were performed on portions of a 10-percent homogenate which had been diluted 50-fold (for other studies) and stored 3 weeks in the frozen state; in our experience such dilution leads to appreciable inactivation of arginase. The value obtained is nonetheless instructive. The activity measured is higher, for example, than that of several of the enzymes of the ornithine-urea cycle in typical ureotelic vertebrates (*8*). Perhaps an approximation of the original activity can be made by repeating this assay on the frozen tissue over a period of several months in order to establish the rate of inactivation.

While we have as yet no information on activities of the enzymes of the uricolytic route for production of urea, we conclude that at least a portion of the urea found in liver tissue of the coelacanth could have been formed enzymically in the living animal from L-arginine. Control experiments performed with rat liver frozen for 14 months indicated little, if any, autolytic production of urea. We thus conclude that the urea measured in the liver of the coelacanth was present when the animal was captured and immediately frozen.

Essentially the same results as those obtained with the coelacanth liver (frozen 6 months) were obtained with bonnethead shark (*Sphyrna tiburo*) liver frozen 5 months (found: 1.4 percent urea by weight, corresponding to the percentage of urea in fresh liver of this shark).

Whether ureotelic or not, all fish appear to contain liver arginase (*19*). Thus, a critical experiment performed on a water homogenate of coelacanth liver was that for another of the ornithine-urea cycle enzymes—ornithine carbamoyltransferase (*11, 18*). Criteria for establishing the presence of this enzyme in coelacanth liver are given in Table 3 and Fig. 1. The relation between product and protein concentration was linear and it was also nearly so with time (for up to a 30 percent conversion of substrate). Stoichiometry of the reaction at 1, 2, and 3 μmole of L-ornithine and carbamoylphosphate was also demonstrated in other experiments. The enzyme appears to be nearly completely specific for the L-ornithine rather than for D-ornithine. A value of 25 μmole of citrulline formed per minute per gram of liver was found for ornithine carbamoyltransferase (mean value from experiments on protein concentration and time). This value is about the same as that found for the same enzyme in *Protopterus* liver (*17*), but it is lower than that for liver of various sharks in Galveston waters (*20*).

The presence of ornithine carbamoyltransferase in coelacanth liver suggests that a route exists for the synthesis of carbamoylphosphate in coelacanths.

We conclude that urea, formed in part at least by enzymes of the ornithine-urea cycle, serves an osmoregulatory function in the coelacanth as it does in elasmobranchs.

Information available concerning the occurrence of a physiologically active ornithine-urea cycle in fishes is summarized in Fig. 2. The designations of the occurrence or absence of the cycle in the various major groups are based in some cases on a limited examination of species and are subject to modification as more information becomes available. The evolution of Actinopterygii and Sarcopterygii from a common ancestor seems to have marked a point at which the potentiality for formation of urea by the ornithine-urea cycle underwent genetic modification.

Table 3. Ornithine carbamoyltransferase activity of coelacanth liver homogenate. The complete system contained: 20 μmole of L-ornithine, *p*H 8.1; 20 μmole of dilithium carbamoylphosphate [Sigma Chemical Co., St. Louis, Mo., Lot No. 24B-5030 (corrected for verified purity of 90 percent)]; 90 μmole glycylglycine (Na) buffer, *p*H 8.3; 0.40 ml (containing 0.93 mg protein) of a 10 percent liver homogenate prepared in water and diluted fourfold in 0.090*M* glycylglycine (Na) buffer, *p*H 8.3; and water to a final volume of 2.00 ml. Incubation was at 25°C for time interval and condition indicated. Citrulline formation was measured as described (*22*) with 2,3-butandione-2-oxime. Absorbance was read in round cuvets (path length, 1.60 cm) in the Bausch and Lomb Spectronic 20 spectrophotometer. A standard curve was prepared from absorbance readings of citrulline standards incubated in the complete system less homogenate.

Deletions from the complete system	Incubation (min)	Citrulline found* (μmole)
	Experiment 1	
None	10	2.77
None	20	4.27
None	30	5.92
	Experiment 2	
None	30	6.86
None	30	6.58
L-Ornithine	30	0.035
L-Ornithine	30	.025
Carbamoyl-phosphate	30	.005
None (boiled homogenate)	30	.020

* Corrected for urea in the zero-time control.

After submitting our manuscript we were informed by F. B. Grant and G. E. Pickford that the serum urea concentration of this coelacanth specimen resembled that of liver and was in the range 324 to 381 μmole per milliliter. Their findings (obtained independently) and their conclusions support our extrapolated conclusion that urea serves an osmoregulatory function in the coelacanth. Thus liver and serum studies are mutually consistent and supporting (*21*).

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References and Notes

1. K. S. Thomson, *Science* **153**, 999 (1966). The anatomical source books appear to be J. L. B. Smith, *Trans. Roy. Soc. S. Africa* **28**, 1 (1940–1941); J. Millot and J. Anthony, in *Traité de Zoologie*, P.-P. Grassé, Ed. (Masson, Paris, 1958), vol. 13, part 3, p. 2553; ———, *Anatomie de Latimeria chalum-*

nae (Centre National de la Recherche Scientifique, Paris, 1958), vol. 1; the second volume is promised for 1966. A popularized account of coelacanths in given by J. L. B. Smith, *The Search Beneath the Sea* (Holt, New York, 1956). In their paper on lipids of the coelacanth, J. C. Nevenzel, W. Rodegker, J. F. Mead, M. S. Gordon, *Science* **152**, 1753 (1966), provide references to the meager biochemical studies performed on coelacanth tissues.
2. J. Z. Young, *The Life of Vertebrates* (Oxford Univ. Press, London, 1950).
3. We are indebted to Dr. K. S. Thomson and the Peabody Museum of Natural History, Yale University, for making liver tissue available to us through Drs. Clement L. Markert and Edward J. Massaro. This is the same specimen described by Thomson in Ref. *1* (male; 107 cm in standard length; 15.9 kg fresh weight; age about 8 years; caught off Iconi, Grand Comore, 14 March 1966, and frozen immediately). (Catalog No. 1482, Fish Collection, Peabody Museum of Natural History.)
4. H. A. Krebs and K. Henseleit, *Z. Physiol. Chem.* **210**, 33 (1932).
5. H. W. Smith, *Biol. Rev. Cambridge Phil. Soc.* **11**, 49 (1936); E. Baldwin, *ibid.*, p. 247.
6. H. W. Smith, *J. Biol. Chem.* **88**, 97 (1930).
7. M. S. Gordon, K. Schmidt-Nielsen, H. M. Kelley, *Fed. Proc.* **20**, 208 (1961); ——— *J. Exp. Biol.* **38**, 659 (1961); K. Schmidt-Nielsen and P. Lee, *J. Exp. Biol.* **39**, 167 (1962).
8. G. W. Brown, Jr., and P. P. Cohen, *Biochem. J.* **75**, 82 (1960); P. P. Cohen and G. W. Brown, Jr., in *Comparative Biochemistry*, M. Florkin and H. S. Mason, Eds. (Academic Press, New York, 1960), vol. 2, p. 161.
9. G. W. Brown, Jr., *Science* **153**, 1653 (1966).
10. R. M. Archibald *J. Biol. Chem.* **157**, 507 (1945).
11. Enzyme Commission, *Enzyme Nomenclature* (Elsevier, New York, 1965), numbers: urease (E.C. 3.5.1.5), same as urea amidohydrolase; allantoinase (E.C. 3.5.2.5), same as allantoin amidohydrolase; allantoicase (E.C. 3.5.3.4), same as allantoate amidinohydrolase; arginase (E.C. 3.5.3.1), same as L-arginine amidinohydrolase; ornithine carbamoyltransferase (E.C. 2.1.3.3), same as carbamoylphosphate: L-ornithine carbamoyltransferase; formerly also termed ornithine transcarbamylase (*8*).
12. Sigma Type V urease, Sigma Chemical Co., St. Louis, Mo. Lot 30B-647-3.
13. R. J. Block, E. L. Durrum, G. Zweig, *Paper Chromatography and Paper Electrophoresis* (Academic Press, New York, ed. 2, 1958), p. 347.
14. St. J. Przylecki, *Arch. Intern. Physiol.* **26**, 33 (1926); A. Brunel, *Bull. Soc. Chim. Biol.* **19**, 805 (1937).
15. G. Duchateau, M. Florkin, G. Frappez, *Compt. Rend. Soc. Biol.* **134**, 115 (1940); M. Florkin and G. Duchateau, *Arch. Intern. Physiol.* **53**, 267 (1943); R. P. Forster and L. Goldstein, *Science* **153**, 1650 (19[illegible] G. W. Brown, Jr., J. James, R. J. Henderson, W. N. Thomas, R. O. Robinson, A. L. Thompson E. Brown, S. G. Brown, *ibid.*, p. 1653.
16. St. J. Przylecki, *Arch. Intern. Physiol.* **24**, 317 (1924); R. Fosse, A. Brunel, P. de Graeve, *Compt. Rend. Acad. Sci.* **190**, 79 (1930); R. Truszkowski and C. Goldmanówna, *Biochem. J.* **27**, 612 (1933); H. A. Krebs and H. Weil, in *Problems of Biology and Medicine*, O. V. Leontovych, Ed. (Moscow, U.S.S.R., 1935), p. 497 (in German); A. Brunel, *Bull. Soc. Chim. Biol.* **19**, 1683 (1937).
17. H. A. Krebs and L. V. Eggleston, *Ann. Acad. Sci. Fennicae, Ser. A. II Chemica* **60**, 496 (1955), appear to be the first to have reported ornithine carbamoyltransferase (*11*) activity in an elasmobranch (*Scylliorhinus canicula*); see also E. Baldwin, *Comp. Biochem. Physiol.* **1**, 24 (1960); G. W. Brown, Jr., in *International Conference on Taxonomic Biochemistry, Physiology, and Serology*, (Univ. of Kansas, Lawrence, 4–6 Sept. 1962). abstr. 11; G. W. Brown, Jr., in *Taxonomic Biochemistry, Physiology, and Serology*, C. A. Leone, Ed. (Ronald Press, New York, 1964), p. 407; P. P. Cohen and G. W. Brown, Jr., *Proc. Int. Congr. Biochem. 5th Moscow 1961* **3**, 129 (1963); R. L. Joseph, D. C. Watts, E. Baldwin, *Comp. Biochem. Physiol.* **11**, 119 (1964); D. C. Watts and R. L. Watts, *ibid.* **17**, 385 (1966); J. M. Schooler, L. Goldstein, S. C. Hartman, R. P. Forster, *ibid.* **18**, 271 (1966). The assay by Krebs and Eggleston for the enzyme was in reverse of the physiological direction for the synthesis of urea by the ornithine-urea cycle, and they concluded that it represented "a step in the formation of urea in selachian liver." Carbamoylphosphate, chemically synthesiz[illegible] [M. E. Jones, L. Spector, F. Lipmann, *J. Amer. Chem. Soc.* **77**, 819 (1955)], was shown to be the intermediate in the citrulline-synthesizing system of S. Grisolia and P. P. Cohen, *J. Biol. Chem.* **204**, 753 (1953); and R. O. Marshall, L. M. Hall, P. P. Cohen, *Biochim. Biophys. Acta* **17**, 279 (1955). For lungfish see P. A. Janssens, *Comp. Biochem. Physiol.* **11**, 105 (1964); G. W. Brown, Jr., *Science* **149**, 1515 (1965); P. A. Janssens and P. P. Cohen, *Science* **152**, 358 (1966). For Amphibia see G. W. Brown, Jr., in *Physiology of the Amphibia*, J. A. Moore, Ed. (Academic Press, New York, 1964), p. 1; H. Manderscheid, *Biochem. Z.* **263**, 245 (1933).
18. Arginase and ornithine carbamoyltransferase were assayed as described by G. W. Brown, Jr., and P. P. Cohen, *J. Biol. Chem.* **234**, 1769 (1959). Protein was determined by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
19. A. Hunter and J. A. Dauphinee, *Proc. Roy. Soc. Ser. B* **97**, 227 (1924).
20. G. W. Brown, Jr., unpublished observations.
21. G. E. Pickford and F. B. Grant, *Science*, this issue.
22. R. M. Archibald, *J. Biol. Chem.* **156**, 121 (1944).
23. A. S. Romer, *The Vertebrate Body* (Saunders, Philadelphia, ed. 2, 1955).

17 November 1966

Nitrate Ions: Potentiation of Increased Permeability to Sugar Associated with Muscle Contraction

Abstract. *Nitrate ions potentiate twitch tension and enhance the increase in permeability to sugar which occurs in electrically stimulated frog sartorius muscles. However, the potentiating effect of nitrate ions on permeability is not dependent upon an increase in twitch tension. The possible relation of changes in permeability to alterations of the concentration of calcium ions in the cell is discussed.*

Exercise is accompanied in vivo by an increased uptake of sugar by skeletal muscles of rats and dogs (*1*). Changes in permeability to sugar have recently been examined in greater detail in frog sartorius muscles stimulated to contract under various conditions in vitro (*2*). Since nitrate ions augment the strength of contraction of muscles in vitro (*3*) we wondered whether or not these ions would modify the increase in permeability to sugar that is associated with contraction.

We used as frog-Ringer bicarbonate solution a modified (*4*) Krebs-Henseleit solution; in nitrate Ringer's solution, $NaNO_3$ was substituted for all of the NaCl. Muscles were stimulated to contract isometrically in Ringer's solution at 19°C with supramaximal, rectangular, direct-current shocks; the initial rate of penetration of 3-*O*-methyl-D-glucose-H^3 was then measured at 19°C as described previously (*2*, *5*) at a substrate concentration of 8 m*M*. 3-Methylglucose, a nonutilizable sugar, and glucose appear to penetrate the muscle cell membrane by way of the same transport system (*5*, *6*). Permeability to sugar can be measured after stimulation is stopped because the effect persists for several hours (*2*).

When frog sartorius muscles are stimulated at frequencies ranging from 3 to 20 shocks per minute, permeability to sugar gradually increases with time and then levels off at a value proportional to the frequency of stimulation (*2*). In our studies, stimulation of muscles in regular Ringer's solution at 15 shocks per minute produced an increase in permeability that eventually leveled off at a value less than the maximum effect attainable with more intense stimulation. When paired muscles were stimulated at the same frequency in nitrate Ringer's solution, permeability rose more rapidly and attained a significantly higher final value (Fig. 1). Thus, nitrate ions can potentiate the effect of electrical stimulation on membrane permeability to sugar.

Nitrate ions had no effect on permeability to sugar in unstimulated muscles. Moreover, when muscles immersed in regular Ringer's solution were stimulated for 30 minutes at a frequency of 120 shocks per minute, a maximum increase in permeability occurred, and nitrate had no further effect.

The rate of penetration of sugar was 11.7 ± 0.8 μmole per milliliter of cell water per hour (mean ± standard error of the mean) for four muscles stimulated in regular Ringer's solution; the rate for paired muscles stimulated in the presence of nitrate ions was 12.3 ± 0.4. The initial rate of penetration of 3-methylglucose into