Table 5. Cell dimensions of magadiite, kenyaite, SH, and keatite, assuming tetragonal symmetry.

Dimensions	Magadiite*	Kenyaite*	SH*	Keatite†
a, Å	12.620 ± 0.020	12.810 ± 0.054	13.000 ± 0.032	7.456
c, Å	15.573 ± 0.079	19.875 ± 0.094	13.678 ± 0.029	8.604
V, Å^3	2480.2 ± 12.81	3261.5 ± 27.49	2311.4 ± 10.73	478.3

* Data from Tables 2–4. † Data from Shropshire *et al.* (*3*).

months does not change the x-ray pattern of either mineral. Hence the H_2O from H_2O- must not occupy structurally sensitive positions. Baker *et al.* (*6*) in their study of the system $Na_2O - SiO_2 - H_2O$ synthesized the compound $3Na_2O \cdot 13SiO_2 \cdot 11H_2O$, but they did not investigate the compositional range in which magadiite and kenyaite occur.

The geologic implications of the magadiite deposit at Lake Magadi are considerable. Because of its chemical composition and its stratigraphic persistence, (it probably covers over 50 square miles—or 130 km^2), the magadiite layer most likely represents a chemical precipitate from alkaline brines of the precursor of Lake Magadi. Jones *et al.* (*7*) found that present-day Magadi brines contain as much as 1450 parts per million SiO_2. Dilution of such brines by fresh water could precipitate magadiite and the fine lamination may represent annual increments of deposition. During subsequent burial, magadiite remains stable, particularly in the presence of alkaline pore fluids. The formation of kenyaite nodules in the magadiite bed is probably due to a gradual removal of sodium by percolating waters. The presence of laminated chert in the center of the larger nodules indicates that this process continues and may eventually lead to the complete conversion of the magadiite bed to kenyaite and then to a chert bed, still preserving the laminations of the original deposit. SH is not formed in the process because of the high *p*H values involved. I have outlined a mechanism for the formation of laminated chert deposits in the absence of organic activity. Govett (*8*) has recently summarized evidence with respect to the depositional environment of the Precambrian iron formations and has concluded that a lacustrine environment is most probable. The mechanism proposed here for the formation of the chert beds can be extended to include the iron-rich bands. In sodium carbonate lakes silica would be precipitated and iron would be stored in solution during the lower-*p*H, dilute stage, whereas iron would be precipitated and silica stored in the brines during the dryer, high-*p*H stages. The alternation would then simply be governed by rainfall and evaporation. We do not yet fully understand why alkaline lakes form (*9*), but we know that the Na/Ca, CO_2/Cl, and CO_2/SO_4 ratios of the inflow waters must be comparatively high. If sodium carbonate species are the main constituents in the closed-basin waters, evaporative concentration will cause a rise in *p*H well above 9 and as high as 11 (*7*). Volcanic and igneous terranes are the most obvious sources for such inflow waters, and in fact most alkaline lakes, including Lake Magadi, lie in volcanic terranes. Extensive volcanic terranes were probably much more abundant during Precambrian times, and we may postulate that many lacustrine basins then were of the sodium-carbonate-bicarbonate rather than the sodium-chloride type. This provides a simple explanation for the great preponderance of banded iron formations during the Precambrian.

HANS P. EUGSTER

Department of Geology,
Johns Hopkins University,
Baltimore, Maryland 21218

References and Notes

1. J. F. Talling and I. B. Talling, *Int. Rev. Ges. Hydrobiol.* **50**, 421 (1965).
2. B. H. Baker, *Geol. Survey of Kenya* **42** (1958).
3. P. P. Keat, *Science* **120**, 328 (1954); J. Shropshire, P. P. Keat, P. A. Vaughan, *Z. Krist.* **112**, 409 (1959).
4. C. W. Burnham, in *Carnegie Institution of Washington Year Book 1961* (Carnegie Institution, Washington, D.C., 1962), p. 132.
5. P. B. Jamieson, *Nature* **214**, 796 (1967).
6. C. L. Baker, L. R. Jue, J. H. Wills, *J. Amer. Chem. Soc.* **72**, 5369 (1950).
7. B. F. Jones, S. Rettig, H. P. Eugster, in preparation.
8. G. J. S. Govett, *Bull. Geol. Soc. Amer.* **77**, 1191 (1966).
9. B. F. Jones, 2nd Northern Ohio Geological Society Symposium on Salt, Cleveland (1966), p. 181.
10. Fieldwork supported by a grant from the Petroleum Research Fund. Permission to work at Magadi and generous logistic support by the Magadi Soda Company through W. Rouse, General Manager, is gratefully acknowledged. B. F. Jones read the manuscript and provided access to unpublished data and help with both field and laboratory problems. O. P. Bricker, III, made valuable suggestions and read the manuscript.

5 July 1967

Lysosomal Enzyme Inhibition by Trypan Blue: A Theory of Teratogenesis

Abstract. *A mechanism of mammalian teratogenesis involving inhibition of embryotrophic nutrition is suggested and exemplified by the action of trypan blue on pregnant rats. There is evidence for the localization of trypan blue in heterolysosomes of the epithelium of the visceral yolk sac, and our experiments indicate that the dye is an inhibitor of a selection of hydrolytic enzymes present in lysosomal fractions from homogenates of rat visceral yolk sac. It seems likely that trypan blue inhibits the intracellular digestion of embryotroph by the visceral yolk-sac epithelium; the conceptus may therefore be deprived of essential nutrients at critical stages of development.*

Agents which cause congenital malformations in mammalia must owe their activity to an effect upon the embryo, the mother, or the site of exchange between mother and fetus. Investigations of teratogenic mechanisms must therefore be concerned with the identification of both the target organ and the particular injury which it receives. For the majority of teratogens neither of these problems has been resolved, and in some cases the possibility that drug metabolism takes place renders the identity of the active material uncertain.

Certain features of the teratogenic activity of trypan blue and related bisazo dyes suggest that in rodents the site of their action is the visceral yolk-sac endoderm. Of the available evidence, reviewed elsewhere (*1*), the following relevant observations may be cited. Action of trypan blue on the embryo seems unlikely since there is no evidence to suggest that the dye is metabolized and since most authors agree that it cannot be demonstrated in embryonic tissues after administration of a teratogenic dose to the mother (*2*). An indirect action of the dye, mediated by changes in maternal metabolism, is rendered less plausible by the well-documented teratogenic effect of these dyes on chick eggs (*3*, *4*). The yolk-sac endoderm in rodents and chicks actively accumulates trypan blue in large quantities and is the only fetal membrane which does so (*5*, *6*). In the rat, cessation of the teratogenic effect of trypan blue (*7*) coincides with the es-

tablishment of a functional chorioallantoic placenta; from this time the embryo is no longer dependent upon the yolk sac for its nutritional requirements. In the chick, where the yolk sac continues to serve as the principal organ of nutrition, no sharp cessation of activity occurs, and one can produce defects [for example, of the limbs (*3*)] characteristic of later developmental stages.

Since its endodermal cells absorb and degrade embryotroph (*6, 8, 9*), the yolk sac in both birds and rodents is an organ of nutrition. Macromolecules are taken into these cells by endocytosis and come to lie in vacuoles (heterophagosomes) demonstrable by both light and electron microscopy; these structures become associated with hydrolytic enzymes to form composite digestive organelles (heterolysosomes) within which hydrolysis of ingested material presumably takes place (*6, 9*). We have treated rats with trypan blue and demonstrated that the dye is taken up together with embryotroph and eventually comes to lie with it in these digestive vacuoles (Fig. 1). The dye has been seen in similar organelles within the proximal kidney tubules of treated rats (*10*).

When the activities of a selection of lysosomal enzymes were tested in the presence of trypan blue, considerable inhibition was observed. Visceral yolk sacs from near-term rat conceptuses were homogenized in 0.25*M* sucrose with a Teflon-on-glass homogenizer. A fraction rich in lysosomal enzymes was prepared by centrifugation (16,300*g* for 20 minutes) of the supernatant of a homogenate previously centrifuged at 3300*g* for 10 minutes. Figure 2 shows the reduction in activities of acid phosphatase, β-glucuronidase, ribonuclease, and deoxyribonuclease in this fraction in the presence of various concentrations of trypan blue; acid protease, assayed by a modification of Anson's (*15*) method, was also inhibited, but only at concentrations of dye above 1 mg/ml. Similar results have been obtained with lysosomal fractions prepared from rat liver (*16*), and it has been further observed that addition of protein to the assay mixture reduces the degree of inhibition caused by trypan blue. This may account for the high concentration of trypan blue needed to inhibit acid protease, for the substrate of this enzyme is itself a protein. The effect of protein is also relevant to the question of whether trypan blue could inhibit lysosomal enzymes in vivo, since the dye almost certainly enters cells in combination with protein and since no inhibition would take place until it had been removed and inhibitory concentrations of free dye had been reached. However, since lysosomes contain proteases and there is electron-microscopic evidence for a concentration of the dye as phagosomes pass more deeply into the cell, these inhibitory concentrations of free trypan blue are likely to be attained.

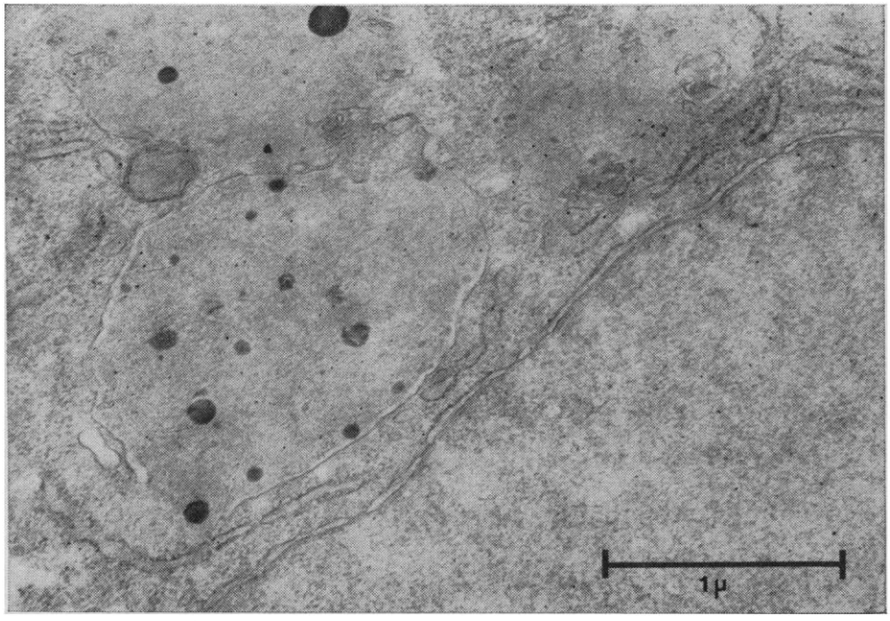

Fig. 1. Supranuclear region of a visceral yolk-sac epithelial cell from a rat treated with trypan blue (75 mg/kg) 24 hours previously. Concentrated granules of dye are confined to deep digestive vacuoles (heterolysosomes) lying near the nucleus.

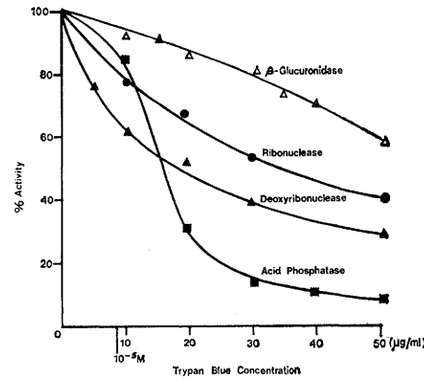

Fig. 2. Inhibition of five lysosomal enzymes of rat yolk sac by trypan blue. Acid phosphatase was assayed at *p*H 4.0 with $10^{-2}M$ *p*-nitrophenyl phosphate used as substrate (*11*); the lysosomal preparation was previously incubated for 10 minutes to inactivate microsomal phosphatases (*12*). β-Glucuronidase was assayed at *p*H 5.0 with $1.25 \times 10^{-3}M$ phenolphthalein mono-D-glucuronide used as substrate (*13*). Ribonuclease was assayed at *p*H 5.0 with ribonucleic acid used (1.5 mg/ml) as substrate (*14*). Deoxyribonuclease was assayed at *p*H 5.0 with deoxyribonucleic acid (1.5 mg/ml) used as substrate (*14*).

Morphological evidence for a localization of injected trypan blue in the digestive vacuoles of the yolk sac, together with the biochemical demonstration of enzyme inhibition in vitro, suggests that the intracellular digestive function of this fetal membrane will be deranged by the dye, with consequent impairment of embryonic nutrition. Histochemical observations of Nebel and Hamburgh (*17*) support this hypothesis. Such a disturbance of yolk-sac function by the dye may be evanescent since cellular regulatory mechanisms might be expected to make good any deficiency, possibly by an increased production of lysosomal enzymes. In fact, certain teratogens are active when applied for very short periods (*18, 19*) and such effect may also operate for bisazo dyes. No information concerning the precise nature of the nutritional deficiency caused by administration of trypan blue is available; this may be a specific factor necessary for normal embryogenesis, but a more general nutritional lack might also be expected to result in a characteristic pattern of malformations, depending upon the developmental stage at which it is operative.

The suggestion that inhibition of embryotrophic nutrition may result in

the production of congenital deformities is widely applicable. Though this communication is principally concerned with teratogenesis induced by trypan blue, it is probable that a variety of exogenous and endogenous inhibitors of enzymes could find their way into the digestive vacuoles of the fetal membranes and exert a similar action. In the rat, digestion of histiotroph is carried out by the visceral yolk-sac endoderm, but in many mammals, including man, this function is largely served by other extraembryonic membranes. Perversion of embryonic nutrition by inhibition of enzymes within lysosomes may therefore involve a variety of placental tissues in various species.

F. BECK
J. B. LLOYD
A. GRIFFITHS

Departments of Anatomy and *Biochemistry, University College of South Wales and Monmouthshire, Cardiff, Wales*

References and Notes

1. F. Beck and J. B. Lloyd, in *Advances in Teratology*, D. H. M. Woollam, Ed. (Logos, London, 1966), vol. 1, p. 131.
2. J. Gillman, C. Gilbert, T. Gillman, I. Spence, *S. African J. Med. Sci.* **13**, 47 (1948); E. E. Goldmann, *Beitr. Klin. Chir.* **64**, 192 (1909); J. G. Wilson, T. H. Shepard, J. F. Gennaro, *Anat. Rec.* **145**, 300 (1963).
3. A. R. Beaudouin and J. G. Wilson, *Proc. Soc. Exp. Biol. Med.* **97**, 85 (1958).
4. F. Stéphan and B. Sutter, *J. Embryol. Exp. Morph.* **9**, 410 (1961).
5. V. H. Ferm and A. R. Beaudouin, *Anat. Rec.* **137**, 87 (1960).
6. F. Beck, *Exp. Cell Res.* **37**, 504 (1965).
7. J. G. Wilson, A. R. Beaudouin, H. J. Free, *Anat. Rec.* **133**, 115 (1959).
8. R. Bellairs, in *Advances in Morphogenesis*, M. Abercrombie and J. Brachet, Eds. (Academic Press, London, 1964), vol. 4, p. 271.
9. F. Beck, J. B. Lloyd, A. Griffiths, *J. Anat.* **101**, 461 (1967).
10. B. F. Trump, *J. Ultrastruct. Res.* **5**, 291 (1961).
11. A. Torriani, *Biochim. Biophys. Acta* **38**, 460 (1960).
12. M. W. Neil and M. W. Horner, *Biochem. J.* **92**, 217 (1964).
13. R. Gianetto and C. de Duve, *ibid.* **59**, 433 (1955).
14. C. de Duve, B. C. Pressman, R. Gianetto, R. Wattiaux, F. Appelmans, *ibid.* **60**, 604 (1955).
15. M. L. Anson, *J. Gen. Physiol.* **20**, 565 (1937).
16. J. B. Lloyd F. Beck, A. Griffiths, L. M. Parry, in *Biological Council Symposium, Interaction of Drugs with Subcellular Components in Animal Cells*, P. N. Campbell, Ed. (Churchill, London), in press.
17. L. Nebel and M. Hamburgh, *Z. Zellforsch. Mikrosk. Anat.* **75**, 129 (1966).
18. L. Pinsky and F. C. Frazer, *Brit. Med. J.* **1960-II**, 195 (1960).
19. R. L. Brent and J. B. Franklin, *Science* **132**, 89 (1960).
20. We thank Profs. J. D. Lever and K. S. Dodgson for their advice and encouragement, and the Spastics Society for a grant in aid of this work. A. G. thanks the Medical Research Council for a research studentship.

20 June 1967

Circadian Clock in Photoperiodic Time Measurement: A Test of the Bünning Hypothesis

Abstract. *A technique has been developed for effectively separating the direct inductive effect of a light signal from its effect on the phase of the rhythm of sensitivity to photoperiodic induction. With this technique it has been shown that a 75-minute pulse of light per day, when appropriately positioned with respect to the circadian activity cycle of the sparrow* Passer domesticus, *is sufficient to produce a response normally produced only by long days. The results cannot be interpreted in terms of a requirement of an absolute amount of either darkness or light and offer strong confirmation of Bünning's hypothesis concerning the mechanism of photoperiodic time measurement.*

Photoperiodic effects on organisms are of necessity based on the ability to distinguish one naturally occurring day length from another and to this extent imply the existence of a biological time-measuring system. The hypothesis that the photoperiodic efficiency of any given light-dark cycle depends primarily on which portion of an underlying circadian sensitivity rhythm is illuminated was first developed by Bünning (*1*). The major feature of this hypothesis is the assumption of the existence of such a circadian rhythm of sensitivity to the inductive effects of light. The circadian clock, which controls many biological rhythms (for example, activity), is assumed also to control the rhythm of photoperiod sensitivity. There is by now a body of data which is consistent with this hypothesis. Many of these data are more or less inconsistent with the somewhat vague alternative hypotheses which have in common conceptualization of the photoperiodic time-measuring system as an hourglass which times the length of the dark period (*2*). However, there are in the literature very few direct and critical tests of Bünning's hypothesis (*3, 4*).

The difficulty in testing the Bünning hypothesis arises from its prediction that light will have a dual action in photoperiodic systems (*3*). In addition to its inductive effect (for example, on flower formation or gonadal development) light, according to this hypothesis, must affect the phase of the underlying sensitivity rhythm because this rhythm is coupled to the circadian clock. This means that different light regimens will "position" the sensitivity rhythm differently with respect to the light portion of the cycle. Unless one knows the effect of a light regimen on the phase of the sensitivity rhythm, one does not know which portion of the rhythm is being illuminated and thus cannot predict the inductive effect of the regimen. Therefore, a meaningful test of Bünning's hypothesis is impossible without information concerning both effects of light. Assays of induction abound, but the sensitivity rhythm by its nature cannot be assayed independent of induction. The best one can do is to use some readily measurable overt circadian rhythm (such as locomotor activity) and make the assumption that both it and the sensitivity rhythm are controlled by the same underlying timing mechanism. Further, one must assume that these rhythms maintain a fixed relationship to one another. The behavior of the measured rhythm can then be taken to reflect the behavior of the hypothesized rhythm of sensitivity to photoperiodic stimuli. The number of cases in which a "test" of the Bünning hypothesis has included measurement of such an overt rhythm remains very small, and, as a result, much of the published discussion of this hypothesis is based on, at best, circumstantial evidence.

If some way could be found to separate the two actions of light (for example, discover a light signal which would be photoperiodically inductive but which would not affect the phase of a measured overt rhythm), various critical tests of the Bünning hypothesis would be possible. One such test arises from the prediction that for "long day" responses a single, very short daily light pulse would be inductive if it could be positioned so as to illuminate the appropriate portion of the sensitivity curve.

Using the house sparrow *Passer domesticus* we have made an effective, though far from absolute, separation of the two actions of light and have used this separation in a critical test of the hypothesis. The feasibility of