Presence of a leak pathway. The failure of the system to retain the esterase at the peak level would suggest that a leak pathway was operating in the reverse direction, transporting the enzyme from inside the vesicles into the bathing medium. In the simplest form of a proton symport coupled to a proton pump being driven by the hydrolysis of ATP, Eddy[17,18] proposed that the solute S, enters the vesicles with n equivalents of protons which are expelled by the proton pump. If m equivalents of protons are inside the vesicles, then as the ratio, n/m increases, so does the value of the ratio $[S]_{in}/[S]_{out}$ at equilibrium. This is consistent with our findings that the value of the transport peaks increased with decrease in the pH of the medium. The ratio, $[S]_{in}/[S]_{out}$ at any given proton concentration is dependent on the concentration of the solute outside the vesicles, $[S]_{out}$[19]. The observation that the ratio $[S]_{in}/[S]_{out}$ increased as the enzyme concentration was increased at pH 2 is in agreement with these proposals. The concept of a leak pathway is also supported by measurements of the mass action ratio for the transport process in whole cells which suggested that the process was close to equilibrium[20].

* Present address: Department of Biochemistry, College of Medical Sciences, University of Maiduguri, P.M.B. 1069, Maiduguri, Nigeria. All correspondence should be addressed to G.I. Adoga.

1 Kaback, H.R., Biochim. biophys. Acta *265* (1972) 367.
2 Leach, F.R., and Snell, E.E., J. biol. Chem. *235* (1960) 3523.
3 Yoder, D.C., Beamer, K.C., Cipolloni, P.B., and Shelton, D.C., Archs. Biochem. Biophys. *110* (1965) 336.
4 Shankman, S., Gold, V., Higa, S., and Squires, R., Biochem. biophys. Res. Commun. *9* (1962) 25.
5 Young, E.A., Bowen, D.D., and Friedrich-Diehl, J., Biochem. biophys. Res. Commun. *14* (1964) 250.
6 Pittman, K.A., Lakshmanan, S., and Bryant, M.P., J. Bact. *93* (1967) 1499.
7 Payne, J.W., and Gilvarg, C., Adv. Enzym. *35* (1971) 187.
8 Adoga, G., and Mattey, M., FEMS Microbiol. Lett. *6* (1979) 61.
9 Adoga, G., and Mattey, M., Biochem. Soc. Trans. *8* (1980) 320.
10 Fukumoto, J., Iwai, M., and Tsujisaka, Y., J. gen. appl. Microbiol. *10* (1964) 257.
11 Nagasaki, S., Neumann, N.P., Arnow, P., Schnable, L.D., and Lampen, J.O., Biochem. biophys. Res. Commun. *25* (1960) 158.
12 Zwartouw, H.T., and Westwood, J.C.N., Br. J. exp. Path. *39* (1958) 529.
13 Halperin, M.L., Connors, H.P., Relman, A.S., and Karnovsky, M.L., J. biol. Chem. *244* (1969) 384.
14 Wilhelm, G., Schultz, J., and Hofmann, E., FEBS Lett. *17* (1971) 158.
15 Fraenkel-Conrat, H., in: Methods in Enzymology, vol. 4, p. 247. Eds S.P. Colowick and N.O. Kaplan. Academic Press, London and New York.
16 Christensen, H.N., Adv. Enzym. *49* (1979) 41.
17 Eddy, A.A., Curr. Top. Membr. Transp. *10* (1978) 279.
18 Eddy, A.A., Biochem. Soc. Trans. *8* (1980) 271.
19 Morville, M., Reid, M., and Eddy, A.A., Biochem. J. *134* (1973) 11.
20 Mattey, M., and Morgan, D., Biochem. Soc. Trans. *6* (1978) 426.

0014-4754/85/111402-04$1.50 + 0.20/0

Establishment of a regeneration-specific in vivo bioassay for neurotrophic activity in denervated *Ambystoma* forelimbs[1]

P.M. Barger and R.A. Tassava[2]

Department of Zoology, The Ohio State University, Columbus (Ohio 43210, USA), 23 January 1985

Summary. Use of continuous ^{3}H-thymidine labeling and subsequent assay for cell cycle activity using a novel parameter, mitotic index of a selectively labeled cell population, has led to the development of a regeneration-specific in vivo bioassay for neurotrophic activity. This system is based on the stimulation of cell cycle arrested cells to resume cycling activity after reinnervation in denervated larval *Ambystoma mexicanum* forelimb stumps.
Key words. Regeneration; neurotrophic; bioassay; *Ambystoma.*

Amputated limbs of several species of urodele amphibians fail to regenerate when maintained in a denervated condition[3]. The nervous influence has been hypothesized to be effected through axonal release of a neurotrophic factor (NTF) into the distal limb tip[3,4]. In vivo[5] and in vitro[6] evidence lend support to the existence of a diffusible NTF. Bioassays measuring stimulation of biochemical and/or cell cycle events in denervated limb stumps/blastemas have reported neurotrophic activity associated with dorsal root ganglia[6-8], nerve and brain extracts[5,9-12], fibroblast growth factor[13], and transferrin[14]. The authentication of a putative neurotrophic factor(s) will ultimately require an in vivo bioassay based specifically on stimulation of a regeneration event. We have attempted to develop such an assay with larval *Ambystoma,* one which measures a restimulation of cell cycle blocked cells in denervated limbs.

Unlike denervated adult newt limbs, denervated limb stumps of larval *Ambystoma* regenerate upon subsequent reinnervation without reamputation[15]. There is limited but measurable early cell cycling in denervated limbs (4-6 days post-amputation/denervation) as determined by mitotic index (MI) and pulse labeling with ^{3}H-thymidine (^{3}H-T), but the MI is very low just prior to reinnervation[16-19]. Increases in the blastema MI to control levels occur upon reinnervation[17-19]. The early cycling cells in denervated limbs have been selectively labeled with ^{3}H-T and scored for subsequent mitotic activity with reinnervation[19]. Olsen et al.[19] showed that this labeled population is prevented from cycling after the initial round of activity and is 'rescued' into renewed cycling activity correlated with reinnervation. Therefore, cells which block due to denervation will contribute to blastema formation in response to the reintroduction of a nervous influence. This specific event in the regeneration of a reinnervated limb can be directly characterized as a neurotrophic response. Theoretically, the introduction of the neurotrophic factor into a denervated limb in the continued absence of nerves will produce the same rescue response.

The potential use of the rescue system as an NTF bioassay has previously been compromised by a low percentage of cells labeling with a single ^{3}H-T pulse and an apparent 'loss' of dedifferentiated labeled cells due to label dilution prior to reinnervation[19]. This result provided only a relatively small and lightly-labeled population scored as rescued, a less than satisfactory system for use as a quantitative or qualitative bioassay.

The present study used continuous labeling to increase the percentage of labeled cells at the early cycling time (days 4–6 post-amputation) and to circumvent label dilution through greater cellular incorporation of ^{3}H-T. This system provides a conclusive illustration of cell rescue through reinnervation and may now be suitable for use as a regeneration-specific in vivo bioassay for neurotrophic activity.

Materials and Methods. Larvae of the Mexican axolotl, *Ambystoma mexicanum,* were maintained at 24 ± 1 °C in 50% Holtfreter's solution. The axolotls were raised to 35–45 mm snout-tail tip length in the laboratory by daily feeding of brine shrimp or lean ground beef. Larvae were anesthetized in 0.15% MS222 (ethyl-m-aminobenzoate methane sulfonate) neutralized with 5% sodium phosphate dibasic[8] for all denervations, amputations, and injections.

Both forelimbs of 27 larvae were amputated through the proximal radius/ulna. Left limbs were denervated one day prior to amputation by severing the 3rd, 4th, and 5th spinal nerves at the brachial plexus; right limbs served as innervated controls. Continuous labeling was obtained by repeat i.p. injections of methyl-^{3}H-thymidine (0.2 µCi/0.01 ml/larva/injection; specific activity 60 Ci/mmole; ICN). Beginning on day 4 post-amputation and continuing through day 6, injections were given at 0, 12, 24, 36 and 48 h post-initial injection. Larvae were fixed in toto in Bouin's fixative at 2, 26 and 50 h post-initial injection (days 4–6 post-amputation) as well as on days 8–10 and 12–14 post-amputation (3 larvae/sampling time).

Individual limbs were prepared for routine paraffin histology and sectioned at 8 µm; sections were distributed on four slides such that each slide bore representative sections of the entire limb. One slide was deparaffinized and dipped in Kodak NTB-2 nuclear track emulsion, stored at 4 °C in a lightproof box for 2 weeks, developed in Kodak D-19 developer, fixed and stained with Harris' hematoxylin and eosin. Determinations were made for labeling index (LI) and mitotic index of just the labeled population (MI_{lab}). LI and MI_{lab} are defined as follows:

LI = (No. labeled cells/total No. cells) × 100%

MI_{lab} = (No. labeled mitoses/No. labeled cells) × 100%

Sampling for LI and MI_{lab} was by random grid placement in the dedifferentiation/blastema areas. Only dedifferentiated nuclei were counted. Nuclei were considered labeled with at least five grains/nucleus; background was two grains/nucleus. A range of 400–1000 cells were sampled per limb. A second slide was stained for nerves by the Samuel[20] technique to confirm denervation and to determine the day of reinnervation. The remaining two slides/limb were held in reserve for additional autoradiography or staining.

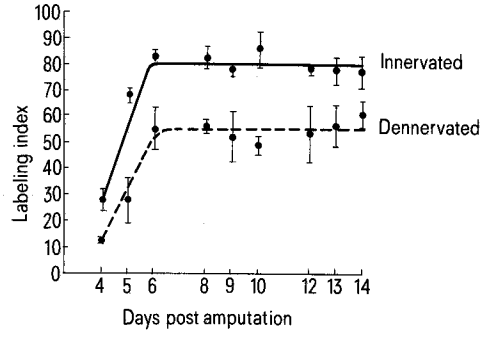

Figure 1. Comparison of labeling indices through time in denervated (–●–) and innervated (—●—) forelimb stumps of larval *A. mexicanum* after continuous labeling with ^{3}H-thymidine from days 4–6 post-amputation. Denervations were performed one day prior to amputation. Each point represents the mean of three limbs ± SD.

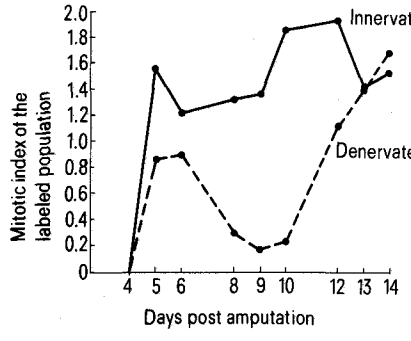

Figure 2. Comparison of mitotic indices of the labeled cell population through time in denervated (–●–) and innervated (—●—) forelimb stumps of larval *A. mexicanum* after continuous labeling with ^{3}H-thymidine from days 4–6 post-amputation. Denervations were performed one day prior to amputation. Each point represents the mean of three limbs.

Results and discussion. The LI data (fig. 1) showed that continuous labeling has the advantage of providing a substantial population of labeled cells to be scored for cell cycle block and rescue. The technique of providing ^{3}H-T to cells at 12-h intervals effectively labels every cell that undergoes DNA syntheses during the labeling period, since the DNA synthetic (S) phase of the cell cycle is much longer than 12 h for axolotl blastema cells (S = 32 h[21], S = 38 h[22]) and therefore no cell can pass through S without being exposed to label. Also, cells which are in the non-DNA synthetic phases of the cell cycle ($G_2 + M + G_1$) at the time of the initial injection will become labeled after 24 h if those cells continue to cycle ($G_2 + M + G_1$ = 8 h[21], 15 h[22]). Figure 1 shows that the LI surpassed 50% in denervated limbs by day 6 after 2 days of continuous labeling, which is considerably greater than the 25% LI obtained in the previous study using only one injection of ^{3}H-T[19]. Note that the pulse LI for the present study was quite low (12.5%) after one injection.

The second advantage of continuous labeling is that it resulted in an effective prevention of any labeled cell loss due to label dilution in the denervated limbs. There is no significant difference between the LI on day 14 and the LI on day 6 (Student's t-test; fig. 1). This is in contrast to the results obtained by Olsen et al.[19] in two experiments where dilution of label due to some cell cycle activity reduced the LI from 24% and 25% on day 4 to 9% and 16% respectively by day 13. Thus only a small population of cells could be scored for rescue. In the present study, continuous labeling led to a greater incorporation of isotope by all cycling cells and dilution of label was circumvented. The large population of labeled cells which persisted through reinnervation allowed for a much more quantitative and reliable demonstration of rescue.

The MI_{lab} data (fig. 2) for denervated limbs provide clear evidence for the three periods of cycling which must occur in order to establish this system as a rescue bioassay: 1) early cycling in response to amputation (days 5–6); 2) subsequent cell cycle inhibition (days 8–10); 3) reinitiation of cycling upon reinnervation (days 12–14).

The cycling observed in denervated limbs (fig. 2) on days 5–6 (MI_{lab} = 0.9%) and the inhibition of cycling by day 10 (MI_{lab} = 0.2%) is consistent with previous results[19]. The MI_{lab} of innervated limbs through the same period (days 5–10) showed no decrease on days 8–10 (fig. 2) and was significantly different from denervated limbs only on days 8, 9 and 10 ($p = 0.05$, Student's t-test). The low MI_{lab} in denervated limbs on days 8–10 was representative of the cycling inhibition which followed the early rounds of cycling on days 5–6[23]. Thus, the early cycling population had blocked in response to denervation and could subsequently be scored for rescue.

Rescue was evident as the MI_{lab} of denervated limbs increased dramatically during the period of reinnervation. Nerve stains indicated that nerves reached the distal limb tip initially on day 9[24]. Previous results[19] indicate that the MI does not increase until

two days after nerves reach the distal limb tip. This result may indicate a G_1 block as the axolotl blastema cell cycle length is 40–53 h[21,22]. In the present study the MI_{lab} increased to control levels upon complete reinnervation (days 12–14). Days 12–14 in reinnervated limbs represented early blastema stages which occured on days 5–7 in innervated limbs. The MI_{lab} for these early blastema stages was virtually identical in reinnervated and innervated limbs (fig. 2). Thus, reinnervation on day 9 can be correlated with the resumption of mitosis in the blocked population by day 12, leading to formation of a blastema. The labeled population contributed significantly to the blastemal cell population as evidenced by the greater than 50% LI on day 14 (fig. 1). The concept of cell rescue using continuous labeling and the MI_{lab} parameter can now be utilized in a valid in vivo bioassay for NTF, one which involves a specific regeneration response – the contribution of previously blocked cells to the reinnervation blastema. The rescue bioassay may not be sensitive enough or quick enough for efficient use as an aid to purifying NTF from nerve or brain homogenates, yet it provides an excellent system for the definitive in vivo test of neurotrophic activity for any candidate molecule.

1 Acknowledgments. We wish to thank the following individuals for comments on the manuscript: M.L. Cecil, D.J. Goldhamer, C.L. Olsen, and B.L. Tomlinson. This work was supported by grants PCM 810 3430 and 831 5428 from the National Science Foundation.

2 To whom reprint requests should be addressed.

3 Singer, M., Rev. Biol. *27* (1952) 169.
4 Singer, M., Ann. N. Y. Acad. Sci. *228* (1974) 308.
5 Lebowitz, P., and Singer, M., Nature *225* (1970) 824.
6 Tomlinson, B.L., Globus, M., and Vethamany-Globus, S., In Vitro *17* (1981) 167.
7 Globus, M., and Vethamany-Globus, S., Devl Biol. *56* (1977) 316.
8 Vethamany-Globus, S., Globus, M., and Tomlinson, B., Devl. Biol. *65* (1978) 183.
9 Foret, J.E., Oncology *27* (1973) 153.
10 Singer, M., Maier, C.E., and McNutt, W.S., J. exp. Zool. *196* (1976) 131.
11 Jabaily, J.A., and Singer, M., J. exp. Zool. *199* (1977) 251.
12 Carlone, R.L., and Foret, J.E., J. exp. Zool. *210* (1979) 245.
13 Mescher, A.L., and Gospodarowicz, D., J. exp. Zool. *207* (1979) 497.
14 Mescher, A.L., and Munaim, S.I., J. exp. Zool. *230* (1984) 485.
15 Butler, E.G., and Schotté, O.E., J. exp. Zool. *88* (1941) 307.
16 Maden, M., J. Embryol. exp. Morph. *48* (1978) 169.
17 Petrosky, N.S., Tassava, R.A., and Olsen, C.L., Experientia *36* (1980) 601.
18 Olsen, C.L., and Tassava, R.A., J. exp. Zool. *229* (1984) 247.
19 Olsen, C.L., Barger, P.M., and Tassava, R.A., Devl Biol. *106* (1984) 399.
20 Samuel, E.P., Anat. Rec. *116* (1953) 511.
21 McCullough, W.D., and Tassava, R.A., Ohio J. Sci. *76* (1976) 63.
22 Wallace, H., and Maden, M., J. Cell Sci. *20* (1976) 539.
23 Note that no MI_{lab} should be seen on day 4 after the 2-h pulse of ^{3}H-T because the length of G_2 is greater than 2 h for axolotl blastema cells (G_2 = 5 h[21,22]).
24 See also Petrosky et al.[17], Olsen and Tassava[18] and Olsen et al.[19].

0014-4754/85/111405-03$1.50 + 0.20/0

Effect of *Plasmodium berghei* infection on benzoic acid metabolism in mice

T.S. Emudianughe*, Q.D. Bickle, M.G. Taylor and B. Andrews

Department of Clinical Pharmacology, Faculty of Health Sciences, University of Ilorin, P.M.B. 1515, Ilorin (Nigeria), and Department of Helminthology, London School of Hygiene and Tropical Medicine, Winches Farm Field Station, 395 Hatfield Road, St. Albans AL4 OYQ (England), 20 December 1984

Summary. The metabolism of benzoic acid was studied in *Plasmodium berghei* infected mice both in vitro and in vivo. Results of in vitro studies showed a considerable decrease in the ability of the infected liver to detoxify benzoic acid by hippuric acid formation. The in vivo study showed that hippuric acid formation decreases with increasing parasitemia and the emergence of benzoyl-glucoronide. This new pathway stops operating with further increase in parasitemia.
Key words. Mouse liver; malaria infection; benzoic acid detoxification; glucuronic acid conjugation; hippuric acid.

Pathological changes have been shown to occur in tissues of host animals after infection by the malarial parasites. Some of the organs affected are the spleen, kidney, liver and the adrenal glands. The liver shows a number of morphological and biochemical changes, the magnitude of which appear to be related to the severity of the infection. Studies of Mercado and Von Brand[8] and Singer[14] showed that livers of parasitized animal exhibited a decreased ability to synthesize glycogen from exogenous glucose and a reduction in coenzyme A content.

The studies of Rosen et al.[10] have shown changes in hepatic ultrastructure caused by malarial parasite. Electron microscopy has revealed alterations in mitochondrial appearance. Sharma et al.[13] have noticed some changes in the biochemical parameters while Patwari et al.[9] showed alteration in serum aspartate transaminase (Serum AST: SGOT) serum alanine transaminase (Serum ALT: SGPT) and alkaline phosphatase in children with *Plasmodium vivax* malaria.

McCarthy et al.[7] studied the effect of malaria infection on drug-metabolizing enzyme activity. This study was limited to phase I drug metabolism. There are possibilities that malaria infection may not affect the phase II drug-metabolizing enzymes. The metabolism of benzoic acid, a carboxylic acid, involves the conjugation of the acid with glycine to form hippuric acid. At low doses (below 100 mg/kg b.wt of mouse), the main metabolite is hippuric acid while at higher doses, a secondary pathway emerges; conjugation with glucuronic acid. When these two pathways are saturated by a further higher dose, benzoic acid is eliminated as the unchanged compound in addition to the glycine and glucuronic acid conjugates. Benzoic acid is here used as a test compound for the enzymes involved in these two pathways.

Materials and methods. The following compounds were purchased: [carboxyl-^{14}C] benzoic acid (Sp. act. 20 mCi/mmole) (Radiochemical Centre Amersham, UK), coenzyme A (CoA), adenosine triphosphate (ATP), glutathione and glycine, hippuric acid and benzoic acid, β-glucuronidase (Sigma Chemical Co. Surbiton, UK).

Infection of mice. Male TFW mice purchased from a local supplier were infected by caudal vein injection of *plasmodium berghei* (N78-strain). The amount of parasite injected was adjusted in such a way that each animal received approximately 10^6 infected red blood cells.

In vitro studies. Animals were killed by a blow to the head and the livers rapidly excised, weighed and homogenized in 5 vol. of