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Amino-acid absorption by developing herring eggs

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ABSTRACT: ^{14}C-glycine absorption by eggs of the herring *Clupea harengus* from a 2 μM solution at 15 °C depends on the stage of embryonic development. Unidirectional ^{14}C-glycine influx rates are small at early stages: 0.6 ± 0.1 and 0.5 ± 0.1 pmoles egg^{-1}h^{-1} in embryos 5 h and 28 h after fertilization, respectively. They increase drastically about 51 h after fertilization (prior to blastopore closure) to 3.7 ± 0.9 pmoles egg^{-1}h^{-1}. Glycine uptake steadily continues to increase almost until hatching (maximum values = 18.8 ± 2.7 pmoles egg^{-1} h^{-1}), decreasing slightly prior to hatching. Distribution ratios (radioactivity μl^{-1} of egg volume: radioactivity μl^{-1} ambient medium) exceed the equilibrium ratio of 1 between 51 h and 78 h after fertilization, reaching values of 4.7 two days prior to hatching, thus suggesting the presence of a transport mechanism capable of transferring the amino acid against the concentration gradient. Curves for concentration-dependent ^{14}C-glycine and ^{14}C-α-aminoisobutyric acid absorption are very similar; they consist of a linear portion at higher concentrations and a saturable component, indicating a mediated uptake process. Calculations performed by means of amino-acid absorption rates and O_2 uptake data suggest that herring eggs scarcely obtain nutritional benefits from absorption of dissolved amino acids in natural spawning areas.

INTRODUCTION

The ability of soft-bodied marine invertebrates to absorb low molecular weight organic compounds across their body surface from trace amounts present in sea water against concentration gradients of several orders of magnitude has been demonstrated for members of nearly all phyla according to their availability and experimental suitability (Jørgensen, 1976). Whether marine invertebrates actually obtain nutritional profit from these absorption processes is still a matter of recent discussion (Stephens, 1975; Sepers, 1977).

To the authors' knowledge there is as yet no indication of such absorption processes occurring in fish eggs. As a first approach to this problem an analysis of amino-acid uptake by herring eggs was undertaken. Mass incubation of artificially fertilized eggs from Baltic spring-spawning herring, *Clupea harengus,* under controlled laboratory conditions provided sufficient egg material for studies on absorption of glycine at different developmental stages. In addition, the concentration dependent uptake of glycine and α-aminoisobutyric acid and the specificity of the glycine absorption mechanism were investigated.

MATERIAL AND METHODS

Running herring males and gravid females were obtained from catches of local Baltic fishermen. Transport from the catching area to the laboratory and stripping, fertilization, and rearing procedures were identical to earlier published methods (Rosenthal, 1968; Rosenthal & Stelzer, 1970). The eggs were fertilized on April 4, 1977, and reared at constant temperature and salinity conditions (12.5° C, 21.9 ‰ S).

For experiments on amino-acid absorption 25 eggs adhering in single files to small (6 × 6 cm) glass plates were thoroughly freed from debris and separated under a dissecting microscope. Thereafter they were transferred into glass vials of 20 ml volume filled with rearing water. Only 15 eggs per sample were used at later stages (from closure of blastopore onward), when glycine uptake rates had increased considerably.

After temperature equilibration (15° C) the rearing water was carefully removed from the egg-samples and replaced by 5 ml of experimental sea water (20 ‰ S), containing 10–750 nmoles of glycine or α-aminoisobutyric acid (AIB), including 50 nCi of uniformly labeled ^{14}C-glycine or [1–^{14}C] AIB (Amersham). In experiments concerning the specificity of the glycine uptake system, 5 ml of experimental sea water contained 50 nmoles of glycine, including 50 nCi of ^{14}C-label, and additionally 500 nmoles of non-radioactive L-alanine, L-valine, L-aspartic acid, or L-arginine.

For direct comparison of glycine uptake between herring eggs and a member of the invertebrate fauna 7–8 specimens (25 mg fresh weight) of the oligochaete annelid *Enchytraeus albidus* were incubated under conditions identical to those applied to the herring eggs. Further experimental details on amino-acid uptake by *E. albidus* are given by Siebers (1976) and Siebers & Bulnheim (1976).

All experiments lasted 0.5 h and were terminated by removal of water and rinsing the incubated material twice with sea water (20 ‰ S, 15° C). Repeated treatment did not change the amount of absorbed label. After transferring the eggs or worms to counting vials they were deep frozen at –25° C.

Counting of absorbed label was performed in a Tracerlab liquid scintillation counter with quench corrections by internal standard after solubilizing animal material for 1.5 h at 60° C in soluene–350 (Packard) and addition of 10 ml of counting solution (5 g PPO + 0.3 g POPOP l^{-1} of toluol). For about 10 % of samples 200 µl of experimental water were used to calculate the amount of radioactive amino acids available to eggs or worms. In these cases 500 µl of distilled water and 10 ml Insta-Gel (Packard) were added before counting. All investigations were run with 3–7 replicates. Differences in absorption rates between varying experimental conditions were analyzed by means of the t-test.

For uptake experiments natural sea water taken from an area near the island of Helgoland (German Bight, North Sea) was diluted with distilled water to a salinity of 20 ‰ and permanently filtered in circuit in the dark. Immediately before experimental use it was ultrafiltered. Total dissolved amino acids were measured fluorometrically with the fluorescamine reagent (North, 1975; Stephens, 1975). Concentrations ranged from nearly undetectable to about 50 nM.

RESULTS

Glycine absorption during the embryonic development

Absorption of dissolved glycine from ambient sea water by herring eggs depends on the ontogenetic stage of the embryo. Uptake rates are small during the first day following fertilization amounting to 0.6 ± 0.1 pmoles $egg^{-1}\ h^{-1}$ and 0.5 ± 0.1 pmoles $egg^{-1}\ h^{-1}$ when exposed 5 h and 28 h after fertilization, respectively (Fig. 1). They drastically increase shortly before blastopore closure (51 h after fertilization) with an uptake rate of 3.7 ± 0.9 pmoles $egg^{-1}\ h^{-1}$, indicating a switch-on of absorptive capacities. This increase steadily continues until just prior to hatching, reaching values of 18.8 ± 2.7 pmoles $egg^{-1}\ h^{-1}$ at an embryo age of 230 h after fertilization. Immediately before hatching, however, a decline becomes obvious. Absolute values (pmoles $egg^{-1}\ h^{-1}$) at a given external glycine concentration and temperature – salinity regime seem to be specific for an attained developmental stage. All data in Figure 1 except those presented in broken lines refer to eggs from one single female. Eggs from a second female, which had been reared at 5° C for 1 week in order to retard embryonic development, were transferred to rearing temperatures of 12.5° C 2 days prior to glycine uptake analysis. Absorption rates were found almost identical to those of the first group, when compared to eggs of the same embryonic stage.

The concentration of ^{14}C-glycine within the egg volume relative to its exogenous concentration was 0.9 and 1.9 in eggs 51 h and 78 h after fertilization, respectively. The last figure exceeds the equilibrium factor of 1, allowing the assumption of a transport mechanism capable of transferring the amino acid against a concentration gradient. In Figure 1 these values are presented as distribution ratios (radioactivity egg^{-1} : radioactivity μl^{-1} incubation medium after 0.5 h of uptake), taking an egg volume of about 1 µl as calculation basis. Distribution ratios steadily increase during the embryonic development, reaching a final value of 4.7 two days prior to hatching.

Glycine and α-aminoisobutyric acid uptake kinetics

The time course of ^{14}C-glycine uptake from 2 μM solution is shown in Figure 2. Absorption rates are linear with time up to an exposure time of 30 min, beyond which uptake tends to level off. Glycine uptake in relation to ontogenetic stage (see preceding paragraph) was measured at a constant exogenous concentration of 2 µM, which is regarded to be close to total amino-acid concentrations in coastal areas (cf. Jørgensen, 1976; Sepers, 1977). To obtain information on absorption kinetics of glycine and the non-metabolizable analogue α-aminoisobutyric acid, concentration dependent uptake experiments were performed at two embryonic stages (Fig. 3).

While from curve A (5 h after fertilization, after few cleavages) concentration dependency is obvious, curves B (glycine) and E (AIB) (155 h after fertilization, with beginning eye pigmentation) reveal the biphasic nature of amino-acid uptake,

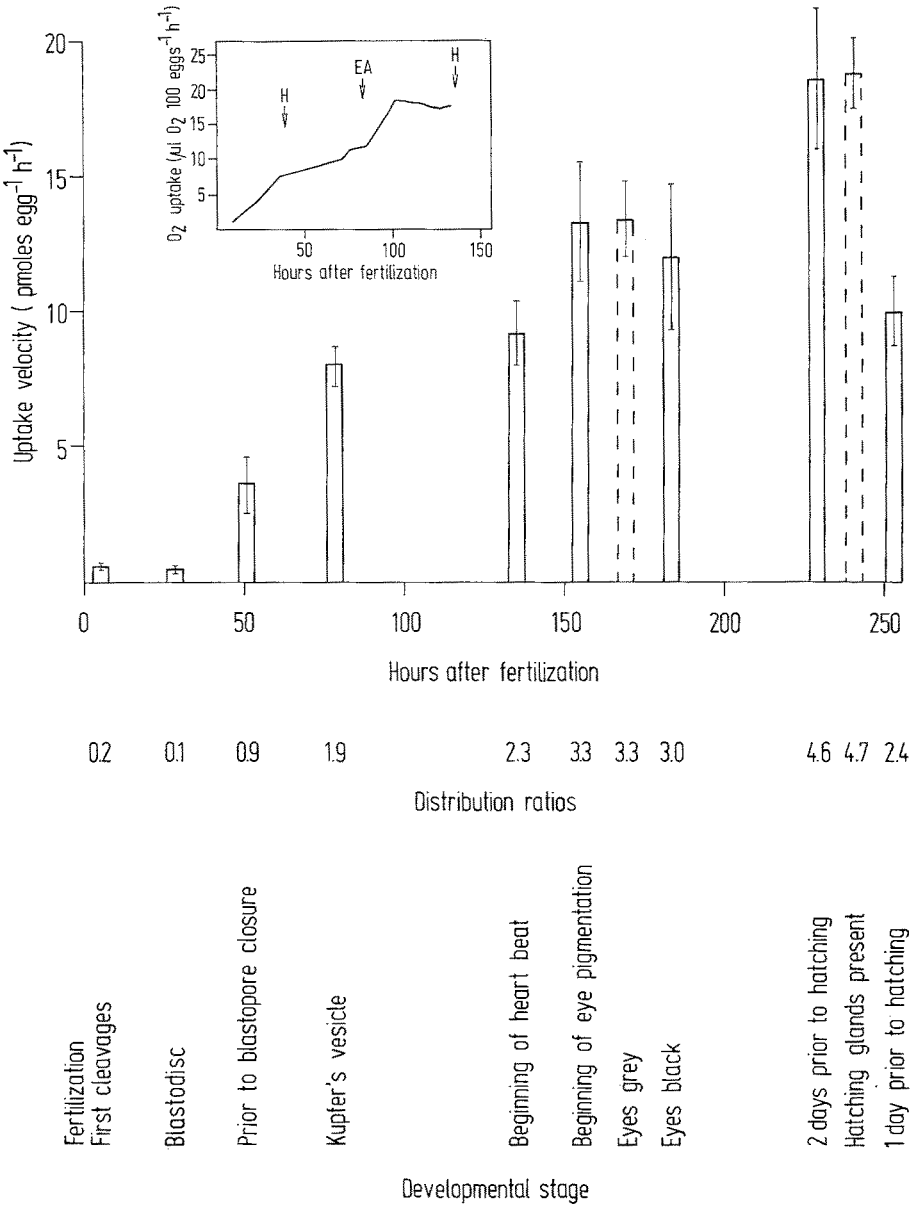

Fig. 1: Uptake of glycine by developing herring eggs (c_{gly} = 2 µM, t = 15 °C, S = 20 ‰, n = 5). All data refer to eggs obtained from the same female except those presented in broken columns. In these cases fertilized eggs were kept at 5 °C to retard embryonic development until 2 days prior to the experiment. The insert (modified and redrawn from Stelzer et al., 1971) refers to O_2 consumption of herring eggs (t = 14 °C, S = 15 ‰) in relation to age (H = beginning of heart activity, E = beginning of eye pigmentation, A = embryonic activity due to increasing rotation of the embryo, H = hatch)

being curvilinear up to about 15 µM and linear above this ambient concentration. This indicates the presence of at least two separate amino-acid uptake systems. The first operates at lower concentrations and reveals saturation kinetics. The second one, which operates at higher concentrations, is linear in relation to ambient concentrations. The curves presented in Figure 3 (B, E) do not reveal whether the non-saturable component results from simple diffusion or belongs to a second facilitated system which is non-saturable within the applied concentration range. The rates

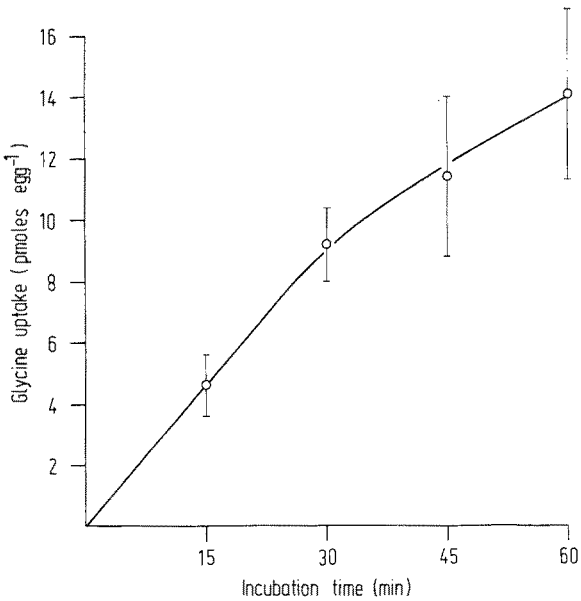

Fig. 2: ^{14}C-glycine uptake by herring eggs in relation to incubation time. The experiment was carried out 135 h after fertilization, when the heart beat started. Standard deviations refer to a sample number of n = 4

of these non-saturable entries were calculated from the slope of the linear portion of the uptake curve (apparent diffusion rate), amounting to 0.43 (glycine) and 0.46 (AIB) pmoles $egg^{-1}\,h^{-1}$ per μM exogenous amino acid. The saturable components of the uptake curves (C = glycine and F = AIB) were obtained after correction of curves B and E for apparent diffusion. Maximum uptake rates (v_{max}) amount to 48 pmoles $egg^{-1}\,h^{-1}$ (glycine) and 65 pmoles $egg^{-1}\,h^{-1}$ (AIB). Transport constants (K_t) – obtained by plotting the reciprocal of uptake velocity against the reciprocal of concentration (Lineweaver-Burk plot) – amount to 6.5 μM (glycine) and 9.0 μM (AIB).

In addition, absorptive capacities of herring eggs were compared to those of the invertebrate *E. albidus*. ^{14}C-glycine and ^{14}C-AIB absorption rates by the oligochaete annelid *Enchytraeus albidus* were inserted in Figure 3. Glycine uptake in *E. albidus* (D) (Siebers, 1976) proceeds about six times faster than in herring eggs, while uptake of AIB (G) is of the same order of magnitude. The differences in kinetic data are remark-

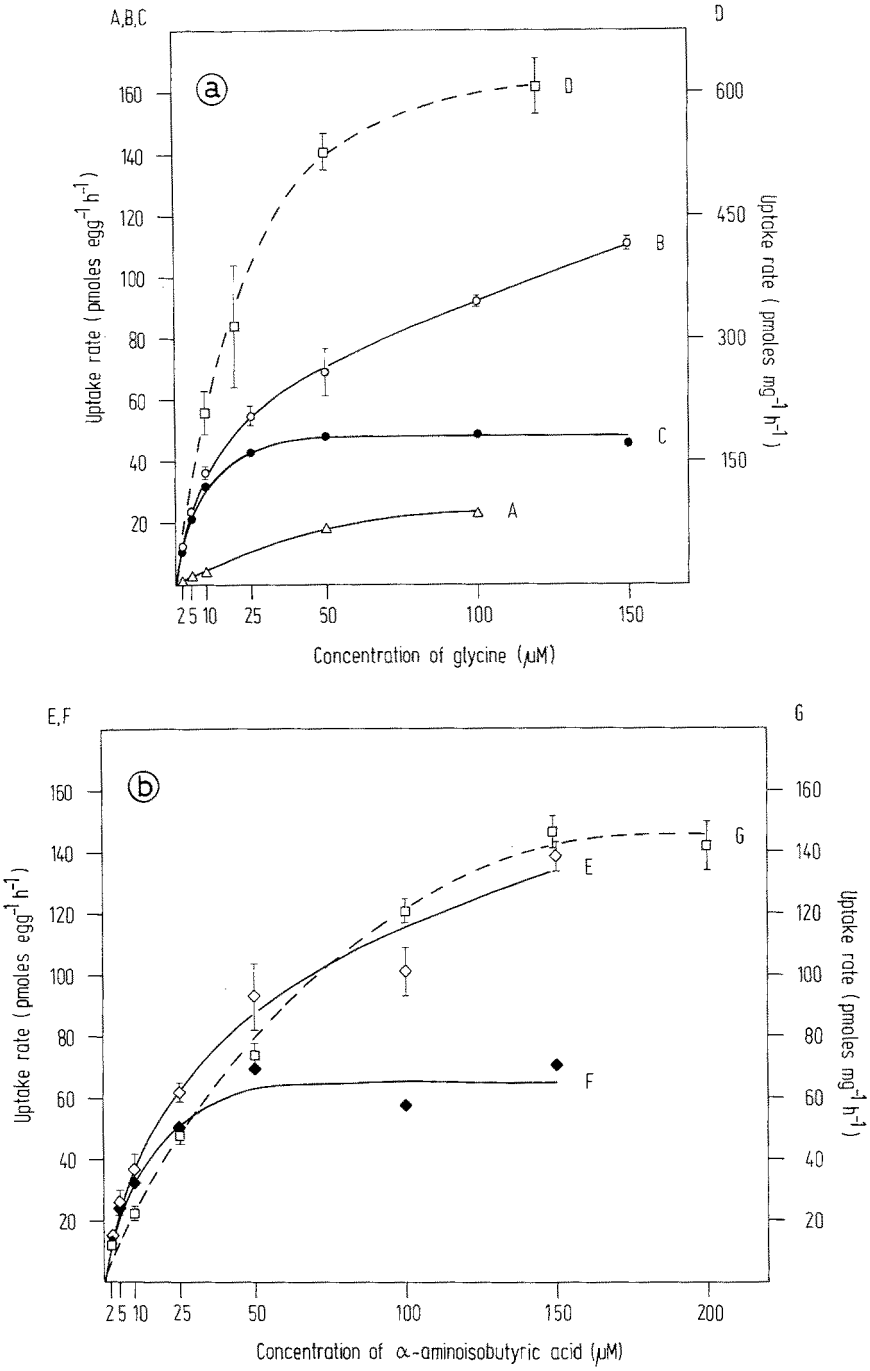

Fig. 3: Concentration-dependent absorption of glycine and AIB by herring eggs and the oligochaete *Enchytraeus albidus* at 15 °C and 20 ‰ S. (a) A, B, C: glycine uptake by herring eggs 5 h (A) and 155 h (B) after fertilization. Curve C shows the saturable component. D: glycine absorption by *E. albidus*. (b) E, F: AIB uptake by herring eggs 155 h after fertilization (E) with the saturable component (F). G: AIB absorption by *E. albidus*. Vertical bars represent standard deviations (n = 4 for herring eggs and n = 6 for *E. albidus*)

able: v_{max}-values obtained for amino-acid uptake by *E. albidus* amount to 700 pmoles $mg^{-1} h^{-1}$ (glycine) and 150 pmoles $mg^{-1} h^{-1}$ (AIB), and K_t-values amount to 20 μM (glycine) and 50 μM (AIB). Both K_t and v_{max} for herring eggs are much smaller than for *E. albidus*. Stephens (1975) reported net glycine uptake for two polychaete species well in the range of the unidirectional glycine influx rates presented for *E. albidus*. The tendency of low K_t- and v_{max}-values in epibenthic and pelagic organisms and higher values in infaunal species (Sepers, 1977) may reflect adaptations of the uptake systems to existing amino-acid concentrations. These are much higher in interstitial waters than in pelagic areas (Stephens, 1975; Sepers, 1977).

Table 1

Effect of 100 µM of charged and uncharged amino acids on ^{14}C-glycine absorption by herring eggs from 10 µM solution. s = standard deviation, referring to n = 4 in controls and n = 3 in test media; uptake rates in test media relative to controls are regarded highly significant (**), if $p < 0.003$ and significant (*), if $0.01 > p > 0.003$ and insignificant (n.s.), if $p > 0.01$

Added amino acid	^{14}C-glycine uptake ± s (pmoles $egg^{-1} h^{-1}$), level of significance	Percentage
Controls	66.0 ± 5.4	100
L-alanine	13.0 ± 1.5 **	19.7
L-valine	32.6 ± 3.6 **	49.4
L-aspartic acid	49.7 ± 7.1 n.s.	75.3
L-arginine	50.4 ± 1.8 *	76.4

In two experiments herring eggs were treated with streptomycin sulphate (20 mg l^{-1}) and penicillin (20 mg l^{-1}) for 4 h and 24 h, in order to test whether bacterial growth on the egg membrane influences the measured glycine absorption. The employed concentrations of antibiotics have been proven to reduce mortality due to bacterial activity in developing garpike eggs, *Belone belone*, from about 30 % to negligible values (Rosenthal & Fonds, 1973). When eggs (eyes grey) were treated with antibiotics for 4 h, glycine uptake from 2 µM solution decreased insignificantly by 4.5 % from 13.4 ± 1.5 (controls) to 12.8 ± 2.0 pmoles $egg^{-1} h^{-1}$ (n = 5). When antibiotics were applied for 24 h (embryos were nearing the end of the eye-pigmentation stage) glycine uptake from 10 µM solution decreased insignificantly by 19 % from 66.0 ± 5.4 (controls) to 53.3 ± 4.4 pmoles $egg^{-1} h^{-1}$ (n = 4). The results reported are not quite conclusive, but confirm that bacterial growth on the egg surface has, if any, only a minor effect on glycine absorption by herring eggs.

Amino-acid absorption capacities of herring eggs reached 13.3 ± 2.2 pmoles of glycine $egg^{-1} h^{-1}$ and 14.4 ± 1.5 pmoles of AIB $egg^{-1} h^{-1}$ from 2 μM solutions at beginning eye pigmentation (Fig. 3). Comparable data were presented by Ahearn & Townsley (1975) for the apodous sea cucumber, *Chiridota rigida*, which was shown to absorb ^{14}C-glycine at a rate of 14.4 pmoles $mg^{-1} h^{-1}$ from an external concentration of 1 µM. This comparison is based on the assumption that the wet weight of one herring egg approximates 1 mg. Scyphistomae of the coelenterate *Aurelia aurita* take up ^{14}C-glycine from 0.8 μM solution (15° C) at rates between 300 and 400 pmoles mg^{-1} dry weight h^{-1} (Shick, 1975), depending on their nutritional state. With respect to the

dry weight calculation basis these values are in the range of results presented for herring eggs.

Specifity of glycine transport

Specificities of amino-acid transporting membranes in relation to the charge of the molecule first became known about three decades ago (Christensen, 1975). Almost independent from cell type, distinct mediations of neutral, basic, and acidic amino acids are observable. From the available set of investigations we chose the inhibition of glycine uptake from 10 µM solution – close to the K_t of glycine influx – by 100 µM of L-alanine and L-valine (neutral amino acids), L-aspartic acid (acidic), and L-arginine (basic). As shown in Table 1, the greatest inhibition of ^{14}C-glycine influx (80.3 %) occurs in the presence of L-alanine, while in the presence of L-valine a reduction by 50.6 % is obtained. Glycine uptake rates are reduced to a much smaller extent after addition of the two charged amino acids L-aspartic acid and L-arginine to the incubation medium. It is not known whether the inhibiting amino acids are taken up at all by herring eggs. However, a specificity of glycine transport for neutral amino acids may also be valid for such a complex structure as a developing herring egg.

DISCUSSION

Uptake of ^{14}C-glycine by herring eggs does not necessarily represent net uptake, but demonstrates unidirectional ^{14}C-glycine influx. The measured uptake values are certainly composed of influx and efflux events. By utilization of the amino acid analogue, AIB, however, true net flux data are obtained, since this amino acid has never been described to be metabolically utilized or incorporated into substances of higher molecular weight. Future experiments will be concerned with the direct analysis of net influxes of naturally occurring amino acids, including distribution of ^{14}C-label within the egg. The good agreement of the concentration-dependent ^{14}C-influx curves for glycine and AIB (Fig. 3) provides some evidence that glycine transport may also result in positive net influxes.

O_2 uptake and glycine absorption similarly increase with ontogenetic age (Fig. 1), suggesting that amino-acid absorption capacities may be associated with increasing embryonic activity. A similar tendency seems to be valid for sea urchin embryos. Uptake of ^{14}C-leucine by developing sea urchin eggs increases from fertilization to hatch with a small retardation between the first metaphase and the subsequent cleavage (Fry & Gross, 1970).

The question whether herring eggs obtain a significant nutritional profit from uptake of amino acids cannot be answered without knowledge of net influx data for natural amino acids. But even if the results obtained represented net influx and no exchange diffusion occurred, uptake rates would be too low to provide more than a few percent of the energy requirements equivalent to the egg's oxygen consumption: calculations are based on the assumption that combustion of 1 µg amino acids (protein)

requires 0.93 μl O_2, of a medium molecular weight of about 100 g $mole^{-1}$ of naturally occurring amino acids, an amino-acid uptake rate of about 15 pmoles (demonstrated for glycine and AIB) $egg^{-1}\,h^{-1}$ from 2 μM solution at 15° C and 20 ‰ S (see p. 469) at the beginning of the eye-pigmentation stage (Fig. 3). At this stage O_2 uptake amounts to about 130 nl $egg^{-1}\,h^{-1}$ at 14° C and 15 ‰ S (Stelzer et al., 1971). The percentage of energy requirement equivalent to O_2 uptake amounts to $15 \cdot 10^2 \cdot 0.93 \cdot 10^2 \cdot 10^{-3} \cdot 130^{-1} = 1.1$ %. We therefore conclude that herring eggs scarcely obtain nutritional profit from absorption of dissolved amino acids in their natural spawning areas, but exclusively depend on the energy content stored as chemical energy in the yolk.

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