PHYLOGENY AND THE DISTRIBUTION OF CREATINE IN INVERTEBRATES [1]

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Studies of the distribution of phosphagens have been interpreted in phylogenetic terms since their inception. Kutscher and Ackermann (1926) suggested that the terms "creatinate" and "acreatinate" might appropriately replace "vertebrate" and "invertebrate." This reflected the opinion that creatine was not merely the characteristic phosphagen of vertebrates but was diagnostic of the sub-phylum.

Most biologists are familiar with the phylogenetic implications drawn from the data of Needham *et al.* (1932) and Baldwin and Needham (1937). These workers concluded that creatine occurred as a phosphagen in some echinoids and ophiuroids as well as in some hemichordates. This, taken together with the supposed diagnostic significance of this compound, was widely accepted as providing biochemical support for the echinoderm ancestry of the vertebrates.

More recent work has led to a reinterpretation of these data so that this biochemical support of phylogenetic orthodoxy now seems less cogent. Yudkin (1954) expressed reservations on this score in his paper on phosphorylation in echinoderms. Creatine has been definitely identified in several annelids and has also been found in sponges and coelenterates (Roche *et al.*, 1957). These authors conclude that this scattered distribution precludes assigning any phylogenetic meaning to the presence of creatine. This view is supported by Ennor and Morrison (1958) in their review and by Kerkut (1960). After reviewing the literature, the latter author states (page 127) that "there is certainly no simple cleavage of the animal kingdom into vertebrates with CP [creatine phosphate] and invertebrates with AP [arginine phosphate]. Instead it is clear that both CP and AP are found throughout the invertebrates." Further (page 127) "one cannot base any pylogenetic speculation on the occurrence of CP or AP since related genera within a class can differ widely in their phosphagens." Finally, he points out that pylogenetic interpretations are also complicated by the recent isolation of several additional substituted guanidines which apparently serve as phosphagens in some invertebrates. These remarks express what seems to be a consensus of opinion, although Baldwin (1963) continues to allude to the distribution of creatine as phylogenetically meaningful.

Despite numerous contributions and even more numerous allusions to this subject, it is clear that the criteria for assigning evolutionary significance to such data have not received as much attention as they deserve. This is in sharp contrast to the care with which the biochemical procedures have been scrutinized. It is also in marked contrast to the care with which the foundations of comparative biochemistry have been discussed in other contexts. Wald (1963) has pointed out in a

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discussion of the distribution of visual pigments that great care must be exercised in attributing phylogenetic meaning to the occurrence of the same molecule in various organisms. He concludes that the presence of retinene in three major phyla as a key constituent of visual pigments is evidence for the unique properties of this molecule rather than evidence for any relation between molluscs, arthropods and vertebrates.

The immediate aim of the present work is to provide quantitative information concerning the occurrence of creatine and guanidinoacetic acid in selected invertebrates. In addition to identifying and estimating these substituted guanidines, we have assayed for transamidinase activity in homogenates of these organisms. Transamidinase is a key enzyme in the apparently unique synthesis pathway of creatine which is found in mammals. It funtions to transfer the amidine group from arginine or another donor to glycine. The guanidinoacetic acid produced by this reaction is then methylated to form creatine.

It became apparent in the course of these observations that the marine invertebrates which were employed were capable of accumulating creatine from extremely dilute solutions. In a number of cases, these same organisms had been reported to be capable of removing glycine from dilute solution in the ambient sea water (Stephens and Schinske, 1961; Stephens, 1963, 1964). This was verified and the observations extended to include uptake of arginine. The capacity of the organisms to take up these compounds made possible additional observations of interest with respect to the synthetic capacities of these forms.

It was hoped that providing quantitative information concerning the occurrence of creatine, supplemented by information concerning the activity of this key enzyme in its synthesis, might add to our understanding of the significance, if any, of the distribution of substituted guanidine compounds among the invertebrates.

Materials and Methods

Organisms were obtained from several sources and shipped to Minnesota where most of this work was carried on. *Glycera dibranchiata* was purchased from "The Maine Bait Company" and presumably had been collected in the vicinity of Newcastle, Maine. *Mercenaria mercenaria* was supplied by a local wholesale fish dealer in Minneapolis. Other forms were obtained from the Marine Biological Laboratory, Woods Hole. Animals were held briefly in moist *Fucus* or in artificial sea water until extracts were prepared or other observation procedures undertaken. Animals incubated with C^{14}-labelled compounds were exposed to dilute solutions of the material in question at 20° C. for the periods indicated.

Creatine was determined according to the procedure described by Van Pilsum *et al.* (1956) and creatine phosphate as indicated by Van Pilsum (1957). The analysis depends on quantitative conversion of creatine to methylguanidine. This procedure gives unequivocal information concerning the presence and amount of creatine, provided the presence of argininosuccinic acid is excluded, as described by Van Pilsum and Halberg (1962). This was done whenever creatine was found. Analyses were carried out using 10% homogenates in 0.6 N perchloric acid which were prepared, neutralized with KOH, and filtered, all at 0° C. One aliquot was analyzed for total creatine. Another was passed through a Dowex-50 column retaining the creatine and passing creatine phosphate. After conversion of creatine

to methylguanidine, this compound was measured colorimetrically by a modified Sakuguchi reaction. The limit of sensitivity of the creatine determination as described is approximately 5 mg. per 100 gm. tissue. Creatine was added to samples of all homogenates as a control procedure. Recovery was quantitative.

Whenever creatine was detected by this procedure, an aliquot of the protein-free extract described above was evaporated to dryness and extracted with petroleum ether to remove lipid. The extracted residue was dissolved in water and desalted by electrodialysis. The material was concentrated and spotted on filter-paper for chromatography. Three different solvent systems were routinely used. They were *n*-butanol-glacial acetic acid-water, 120:30:50; *n*-butanol-pyridine-water, 65:65:65; and isopropyl alcohol-ammonia-water 200:10:20 (Smith, 1960). Location reagents were prepared according to Smith (1960). Sakaguchi reagent was used for locating guanidinoacetic acid and methylguanidine, and diacetyl reagent for creatine.

Transamidinase was determined by the procedure described by Van Pilsum *et al.* (1957) and by a more sensitive modification of this technique (Van Pilsum *et al.*, 1963). Aliquots of a water homogenate (5% by weight) of the organism concerned were incubated with equimolar amounts of canavanine and glycine at pH 7.4. Canavanine is an analogue of arginine. It was used because it is a more effective amidine-group donor than arginine. In most cases, when transamidinase activity was found, the assay was repeated using arginine as a donor. Before and after incubation protein-free filtrates were prepared, using barium hydroxide and zinc sulfate. The guanidinoacetic acid formed was measured in this filtrate, using a modified Sakuguchi reaction. Whenever transamidinase activity was found, aliquots of this filtrate were extracted with petroleum ether and desalted and chromatographed using the solvent systems described above. The limit of sensitivity of this procedure is approximately 50 micrograms of guanidinoacetic acid formed per gram wet weight in six hours of incubation.

We were concerned to report our negative results and attach some significance to them. In order to reduce the probability that negative results for transamidinase activity were the result of some inhibitor in our homogenates, rat kidney homogenate was added as a routine control procedure. The transamidinase activity was recovered in all cases. To check the possibility that guanidinoacetic acid was being formed and entering some further reaction sequence, an aliquot of homogenate was incubated with added guanidinoacetic acid. In all cases where negative results for transamidinase were obtained, the added guanidinoacetic acid was recovered quantitatively. In certain cases the incubation mixtures were analyzed for creatine as well, to exclude the possibility of its synthesis.

Uniformly labelled glycine-C^{14} was supplied to the organisms indicated as dilute solutions in sea water. Creatine-methyl-C^{14} was employed for most of these observations although some initial observations were made using creatine labelled in the carboxyl position. Radioactivity of the ambient medium and appropriate extracts was determined using a thin-window Geiger tube. Data presented are corrected for background and sample thickness. Chromatograms of radioactive compounds using three different solvent pairs were scanned and compared with chromatograms of authentic labelled material. Creatine was also located using the diacetyl reagent in chromatograms of radioactive material.

Results

The organisms used can be divided into three groups based on data obtained concerning the presence of creatine and guanidinoacetic acid and the presence of transamidinase activity. In one group of the invertebrates tested, neither of the substituted guanidines was found nor could transamidinase activity be detected. Detectable levels of transamidinase activity and guanidinoacetic acid were present in a second group but no creatine could be demonstrated. Finally, the third group possessed creatine but no guanidinoacetic acid and no transamidinase activity could be detected. These relations are indicated in Table I. It should be indicated that no selection has been exercised; all animals examined fall into one or another of these three groups.

Table I

Transamidinase activity is expressed as micrograms of guanidinoacetic acid formed per gram of tissue after six hours of incubation. A dash means less than 50 micrograms. The column labelled guanidinoacetic acid refers to the presence of a spot with the R_f value of this compound on a paper chromatogram. A protein-free extract of the organism was used as described in the text. Creatine content is expressed as milligrams per 100 grams wet weight of the organism. A dash means less than 5 mg./100 gm.

Species (phylum)	Transamidinase activity	Guanidinoacetic acid	Creatine
Glycera dibranchiata (Annelida)	—	—	500
Diopatra cuprea (Annelida)	—	—	100–200
Saccoglossus kowalevski (Hemichordata)	—	—	30
Styela carnea (Chordata)	—	—	15–35
Metridium dianthus (Cnidaria)	100	+	—
Nereis virens (Annelida)	250	+	—
Lepidonotus squamatus (Annelida)	900	+	—
Mercenaria mercenaria (Mollusca)	300	+	—
Amphitrite ornata (Annelida)	—	—	—
Golfingia gouldii (Sipunculoidea)	—	—	—
Spisula solidissima (Mollusca)	—	—	—
Thyone briareus (Echinodermata)	—	—	—
Leptopsynapta inhaerens (Echinodermata)	—	—	—
Ciona intestinalis (Chordata)	—	—	—
Amaroucium constellatum (Chordata)	—	—	—

Creatine phosphate was demonstrated qualitatively in the four animals which showed detectable creatine. In *Glycera*, 285 mg. per hundred grams wet weight, or 57% of its creatine, was in the form of creatine phosphate. Since it was somewhat unexpected to find that the organisms which possessed creatine showed no transamidinase, additional observations were undertaken. Efforts to identify creatine in protein-free extracts of *Nereis* and *Mercenaria* by chromatography were negative. Five per cent homogenates of *Glycera, Diopatra, Styela* and *Saccoglossus* were incubated with canavanine and glycine at pH 7.4 and examined for possible creatine formation both colorimetrically and chromatographically. No creatine synthesis could be demonstrated.

Saccoglossus and *Glycera* were exposed to dilute solutions of uniformly labelled arginine-C^{14} and glycine-C^{14} for periods of one to four hours. They were then

maintained for 24 to 96 hours in sea water and sacrificed. A perchloric acid extract of the animals treated in this way showed considerable radioactivity as a result of the accumulation of these amino acids from the surrounding medium.

The extracts were desalted and defatted, chromatographed, and passed through the chromatogram scanner. Clear peaks of radioactivity were present at the R_f values of authentic samples of arginine or glycine, respectively. Creatine could be located using diacetyl reagent. No radioactivity was found in any experiment of this sort associated with the creatine. Thus, the organisms could not be demonstrated to synthesize creatine from appropriate precursors by this approach, during periods as long as four days.

Several of these species were also examined with respect to their ability to accumulate creatine from dilute solution in the surrounding sea water. *Saccoglossus, Glycera, Nereis* and *Mercenaria* all exhibited this ability. Details will be presented for *Glycera.*

When individual bloodworms were exposed to creatine-C^{14} labelled in the carboxyl position at concentrations as low as 10^{-7} moles per liter of sea water, the labelled material was accumulated. Chromatograms showed radioactivity at the same R_f as that exhibited by authentic creatine. Creatine labelled in the methyl position behaved in the same way.

The relation between weight and rate of uptake was exponential (*cf.* Stephens, 1963). For 24 worms ranging in weight between 2 grams and 11 grams, a plot of the log of wet weight *versus* the log of rate of uptake gave a reasonable fit to a straight line. The least squares regression line had a slope of 0.49 ± 0.05. The correlation coefficient was 0.907. Hence, rate of uptake was taken to be linearly related to the square root of wet weight. This relation was used to reduce subsequent data to a form which permitted comparison among animals of different weight. An effort was made to minimize size variations.

It seems likely that accumulation occurred directly across the body wall since rate of uptake by individuals ligated at the head and tail was not significantly different from that shown by normal individuals. The rates in arbitrary units were 1.26 ± 0.29 ($N = 6$) and 1.41 ± 0.35 ($N = 6$), respectively.

Rate of accumulation was measured at concentrations ranging from 2×10^{-5} moles per liter to 10^{-3} moles per liter. The reciprocal of the measured rate was plotted against the reciprocal of ambient concentration. The resulting points fell along a straight line, permitting estimation of maximum rate of uptake and the concentration at which uptake is half maximal. The procedure is analogous to estimation of V_{max} and K_m by using a Lineweaver-Burk plot. Of course no implication that the process is enzymatic is intended. However, the fit of the data to a straight line suggests an adsorptive rate-limiting step at higher ambient concentrations. V_{max} for creatine uptake is 1.05 ± 0.15 micromoles per hour per gram for worms weighing 7.35 ± 0.59 grams ($N = 12$). K_m is approximately 2.0×10^{-4} moles per liter of creatine.

Creatine was not readily lost to the environment by these worms nor was it easily exchanged. After 96 hours, radioactive creatine labelled with C^{14} in either the methyl or the carboxyl group could still be demonstrated. Chromatograms verified that radioactivity was still in the form of creatine. Insufficient quantitative data are available to calculate a rate of loss of labelled creatine but it is apparently quite slow since no marked decline in activity was found at the end of this period. A

group of 24 animals was exposed to creatine-C^{14} for 60 minutes, rinsed in sea water for five minutes, and placed in 50 ml. of sea water containing 10^{-4} moles per liter of unlabelled creatine No radioactivity above background was found at the end of two hours. Extracts of several worms selected at random ranged between 1000 and 2000 cpm. per gram. Hence, exchange of creatine with the environment appears to proceed extremely slowly.

Less data are available for *Saccoglossus,* but it removed creatine from dilute solution rapidly. *Nereis* and *Mercenaria* also showed this capacity. Some data are available which suggest that creatine-C^{14} was lost or degraded in *Nereis* over a period of 48 to 96 hours after its accumulation. Extracts made 48 hours after exposure to radioactive creatine showed only about one-third of the activity exhibited by extracts made immediately after exposure. Only four specimens were measured but this result contrasts with the failure to demonstrate depletion in *Glycera.* Chromatography established that radioactivity still resided in creatine when carboxyl-labelled or methyl-labelled material was used. However, there was an additional radioactive spot in the case of the creatine-methyl-C^{14}. This has not been firmly identified, but suggests degradation of creatine before its elimination.

An effort to detect creatine in sea water samples collected in regions where *Glycera* is present was unsuccessful. This implies that concentrations are less than 1 mg. per liter if creatine is present.

Discussion

The distribution of creatine, guanidinoacetic acid, and transamidinase activity found in the present investigation is particularly intriguing in those invertebrates which exhibit creatine: none has any demonstrable transamidinase. Several antecedent possibilities can be indicated. It may be that transamidinase is present but activities are too low to be demonstrated by the present procedure. Alternatively, creatine may be synthesized by some pathway which does not involve the transfer of the amidine group to glycine in the usual fashion. As yet another possibility, one might speculate that demonstrating invertebrate transamidinase activity requires conditions which are not provided in these experiments. None of these possibilities seems likely. It must be borne in mind that no selection of cases has occurred. All the organisms which show creatine fail to show transamidinase activity. It would be unusual to pick four phylogenetically scattered organisms, all of which just happen to have very low levels of transamidinase despite having considerable amounts of creatine in their tissues. The uniform presence of transamidinase activity in homogenates of organisms having guanidinoacetic acid indicates that there is no widespread set of highly specific requirements for demonstration of transamidinase in invertebrates. Finally, the hypothesis that some new pathway of synthesis is involved receives no support from the attempts to produce labelled or unlabelled creatine *in vivo.* This is not to say that these possibilities can be totally discarded. However, none of them receives any support from our observations and none of them has very high antecedent probability.

Another explanation can be advanced. The invertebrates which show creatine may acquire it from some exogenous source. This hypothesis has some obvious attractions and is supported by our data. If creatine were acquired by marine invertebrates as part of their food or by accumulation from dilute solution in their

environment, then a more satisfactory account of the extremely scattered distribution of this compound among invertebrates could be offered. Organisms such as *Glycera* might be thought of as employing creatine as a phosphagen in an adventitious fashion. Presumably, creatine might be available to any organism feeding on certain diets or adapted to accumulate it. However, only some organisms would develop the phosphoryl transfer enzyme necessary to employ it as a phosphagen. If this were the case, neither the presence of creatine as an isolated compound nor its use as a phosphagen would have phylogenetic significance.

In support of this possibility, it can be noted that one would expect to find no transamidinase associated with creatine since by hypothesis, it would be acquired exogenously rather than synthesized. One would also expect to find other organisms in the same general area which were capable of acquiring creatine but which eliminated it without using it as a phosphagen. This appears to be the case in *Nereis* and *Mercenaria*. Finally, this permits some reconciliation of earlier views concerning the phylogenetic significance of creatine distribution with our more recent information concerning the occurrence of this compound among invertebrates. It seems very reasonable to demand that if a compound is to carry the phylogenetic freight that has been borne by creatine in past decades, it should meet more rigorous standards than are currently stipulated. In particular, one should ask for a demonstration of some continuity in the pattern of synthesis and degradation of the material in question. This might be compared to the demand that morphologically comparable units or homologues must exhibit the same larger pattern and not merely identity of structure in some self-contained sense. Outstanding examples come to mind in the discussion of evolution and protein structure (Margoliash, 1963).

No creatine was found in the habitat of these organisms. It is true that the search was casual. It is also true that one can think of potential sources. Creatine is an excretory product in some marine fishes (*cf.* Prosser and Brown, 1961). It has also been reported as present in soils from various locations (Schreiner and Skinner, 1912) and as a constituent of a number of plants (Guggenheim, 1958). It is not *prima facie* unreasonable that small amounts of creatine might be present in solution in the sea water of estuarine locations nor is it unreasonable that creatine might be present in detritus or some other dietary constituents. However, this remains to be demonstrated.

The present observations indicate the need for reexamination of the distribution of creatine and transamidinase to provide the data necessary for a more sophisticated approach to the possible phylogenetic significance of phosphagen distribution. We support the position that the distribution of creatine among invertebrates as an isolated observation probably has no phylogenetic implications. However, this should not be taken as a denial of the possibility of biochemical support for phylogenetic conclusions based on studies of phosphagen distribution. One should rather demand that conclusions concerning phylogeny be based on more adequate criteria defining homology at the molecular level. Put in another way, our present information permits interpreting the presence of creatine in the invertebrates we have examined as adventitious and, therefore, irrelevant to a phylogeny which may well be reflected in the distribution of creatine and the supporting biochemical matrix in which it is embedded in vertebrates.

Summary

1. Fifteen species of invertebrates from seven phyla were examined for the presence of creatine, guanidinoacetic acid, and transamidinase.

2. None of the animals which possessed creatine had detectable levels of transamidinase. All animals which possessed guanidinoacetic acid also possessed transamidinase activity. A third group of invertebrates was formed of those species in which neither of the guanidine compounds nor transamidinase could be demonstrated.

3. Attempts using various procedures to obtain synthesis of creatine from appropriate precursors in those organisms in which it was found were uniformly negative.

4. Accumulation of creatine from very dilute solution in the ambient medium was demonstrated for all of the invertebrates in which creatine was found.

5. In *Glycera* and *Saccoglossus,* both of which possess creatine, creatine-C^{14} obtained from the ambient medium persists as creatine for at least 96 hours.

6. Other organisms which do not normally possess creatine also showed the ability to accumulate it from dilute solution. Evidence is presented that such animals eliminate creatine and may degrade it in the process.

7. These findings are discussed and their possible significance for phylogeny considered. It is suggested that the creatine found in the invertebrates examined here may be exogenous in origin. Thus, its presence may be adventitious and its use as phosphagen opportunistic.

LITERATURE CITED

Baldwin, E., 1963. Dynamic Aspects of Biochemistry (4th edition). Cambridge University Press.

Baldwin, E., and D. M. Needham, 1937. Comparative biochemistry of muscular and electrical tissues. *Proc. Roy. Soc. London, Ser. B,* **122**: 197–219.

Ennor, A. H., and J. F. Morrison, 1958. Biochemistry of the phosphagens and related guanidines. *Physiol. Rev.,* **38**: 631–674.

Guggenheim, M., 1958. Die biogenen Amine in der Pflanzenwelt. *Handbuch der Pflanzenphysiologie,* **8**: 889–988.

Kerkut, G. A., 1960. Implications of Evolution. Pergamon Press, New York.

Kutscher, F., and D. Ackermann, 1926. Vergleichend-physiologische Untersuchungen von Extrakten verschiedener Tierklassen auf tierische Alkaloide eine Zusammenfassung. *Zeitschr. Biol.,* **84**: 181–192.

Margoliash, E., 1963. Primary structure and evolution of cytochrome c. *Proc. Nat. Acad. Sci.,* **50**: 672–679.

Needham, D. M., J. Needham, E. Baldwin and J. Yudkin, 1932. A comparative study of the phosphagens with some remarks on the origin of vertebrates. *Proc. Roy. Soc. London, Ser. B,* **110**: 260–294.

Prosser, C. L., and F. A. Brown, Jr., 1961. Comparative Animal Physiology. W. B. Saunders Company, Philadelphia.

Roche, J., N. Thoai and J. L. Hatt, 1957. Sur la présence de créatine chez les Invértébrés et sa signification biologique. *Biochim. Biophys. Acta,* **24**: 514–519.

Schreiner, O., and J. J. Skinner, 1912. Nitrogenous soil constituents and their bearing on soil fertility. *USDA Bur. Soils Bull.,* **87**: 5–84.

Smith, I., 1960. Chromatography and Electrophoretic Techniques. Vol. 1: Chromatography. Interscience Publishers, Inc., New York.

Stephens, G. C., 1963. Uptake of organic material by aquatic invertebrates. II. Accumulation of amino acids by the bamboo worm, *Clymenella torquata. Comp. Biochem. Physiol.,* **10**: 191–202.

Stephens, G. C., 1964. Uptake of organic material by aquatic invertebrates. III. Uptake of

glycine by brackish-water annelids. *Biol. Bull.,* **126**: 150–162.

STEPHENS, G. C., AND R. A. SCHINSKE, 1961. Uptake of amino acids by marine invertebrates. *Limnol. Oceanogr.,* **6**: 175–181.

VAN PILSUM, J. F., 1957. Creatine and creatine phosphate in normal and protein-depleted rats. *J. Biol. Chem.,* **228**: 145–148.

VAN PILSUM, J. F., AND F. HALBERG, 1962. A method for the determination of argininosuccinic acid in human urine. *Amer. J. Mental Deficiency,* **67**: 82–89.

VAN PILSUM, J. F., D. A. BERMAN AND E. A. WOLIN, 1957. Assay and some properties of kidney transamidinase. *Proc. Soc. Exp. Biol. Med.,* **95**: 96–100.

VAN PILSUM, J. F., R. P. MARTIN, E. KITO AND J. HESS, 1956. Determination of creatine, arginine, guanidinoacetic acid, guanidine and methyl-guanidine in biological fluids. *J. Biol. Chem.,* **220**: 225–236.

VAN PILSUM, J. F., BEATTA OLSEN, DORRIS TAYLOR, T. ROZYCHI AND J. C. PIERCE, 1963. Transamidinase activities, *in vitro,* of tissues from various mammals and from rats fed protein-free, creatine-supplemented and normal diets. *Arch. Biochem. Biophys.,* **100**: 520–524.

WALD, G., 1963. Phylogeny and ontogeny at the molecular level. *Proc. 5th Int. Congr. Biochem.,* **III**: 12–51.

YUDKIN, W. H., 1954. Transphosphorylation in echinoderms. *J. Cell. Comp. Physiol.,* **44**: 507–518.