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STUDIES ON DEOXYNUCLEOSIDIC COMPOUNDS

I. A MODIFIED MICROBIOASSAY METHOD AND ITS APPLICATION TO SEA URCHIN EGGS AND SEVERAL OTHER MATERIALS*

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SUMMARY

1. By modifying HOFF-JØRGENSEN's microbiological assay method for deoxynucleosides, *viz.* by digesting samples to be assayed with a snake venom enzyme, it was possible to detect a new group of deoxynucleosidic compounds. This group of deoxynucleosidic compounds, differing from either simple deoxynucleosides or deoxynucleotides, were tentatively designated as "masked" deoxynucleosidic compounds.

2. "Masked" deoxynucleosidic compounds were detected in various tissues such as sea urchin eggs and embryos and mammalian tissues including tumors.

3. The relative amounts of "masked" deoxynucleosidic compounds compared with DNA or simple deoxynucleosidic compounds in eggs and early embryos were markedly higher than those in adult tissues. In this connection, the biological significance of "masked" deoxynucleosidic compounds was discussed.

Abbreviations: DNA, deoxyribonucleic acid; PCA, perchloric acid.

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INTRODUCTION

It is generally assumed that DNA carries the genetic information of the living systems, and the physical and chemical properties as well as the biological role of this macromolecule have been studied extensively. However, relatively little is known about the natural occurrence of acid-soluble deoxynucleosidic compounds, which may play some role in the active metabolism of DNA and cell proliferation.

To perform an experiment on the nature and role of these deoxynucleosidic compounds, it seems desirable to select a biological system in which rapid multiplication and active DNA metabolism take place. Further, an analytical method is required by which a minute amount of deoxynucleosidic compounds can be specifically determined in the presence of a relatively large amount of ribonucleosidic compounds.

In this study, unfertilized eggs and early embryos of sea urchin (*Hemicentrotus pulcherrimus*) were used as the main experimental material. The unfertilized eggs are thought to be in the particular physiological condition preceding the rapid cell division (cleavage) that ensues soon after fertilization[1]. On the other hand, embryos of early developmental stages are regarded as representative of the system in which rapid cell multiplication is under way.

As an analytical method, we employed the microbiological method, originally devised by HOFF-JØRGENSEN[2,3] for the analysis of deoxynucleosides and deoxynucleotides; it has been found very useful because of its high specificity and sensitivity.

During the progress of this investigation, an interesting fact came to our notice. If pretreatment by snake venom digestion were carried out prior to the original microbiological assay, hitherto undescribed deoxynucleosidic compounds were found to be detectable in acid-soluble extracts of eggs and embryos of sea urchin, toad embryos, several animal tissues including tumors, and microorganisms.

In this paper, details of our modified microbiological procedure and several results obtained by applying this procedure to eggs and embryos of the sea urchin and tissues of the rat and mouse are described.

A preliminary report has appeared elsewhere[4].

MATERIALS AND METHODS

Eggs and embryos of the sea urchin. Samples of mature unfertilized eggs of the sea urchin, *Hemicentrotus pulcherrimus*, were prepared as follows. The spawning was induced by introducing an isotonic KCl solution into the body cavity. After filtration through gauze, the eggs were exposed to HCl–sea water (pH 4.5–5.5) to remove the jelly coat, and washed several times with filtered sea water. The eggs were further washed with isotonic KCl, frozen with dry ice–ethanol and lyophilized. Only eggs from the lots showing fertilization reaction upon insemination were used for the experiment.

To obtain embryos of a given developmental stage, the jelly-less eggs were inseminated with sperm suspension prepared by diluting the "dry sperm" ten thousand-fold with sea water, and cultured at room temperature after washing several times with isotonic KCl and sea water to remove excess spermatozoa. Care was taken to avoid crowding of the eggs or embryos during culture. The embryos that reached the desired stage of development were collected by centrifugation, washed with isotonic KCl, frozen and lyophilized.

The lyophilized samples of eggs and embryos were defatted with ethanol–ether (1:3) to facilitate the subsequent extraction with PCA. The defatting procedure did not remove any appreciable amount of deoxynucleosidic compounds.

Rat tissues and tumors. Tissues of the rat and mouse were obtained from decapitated animals as quickly as possible and immediately homogenized with cold 0.2 *N* PCA or frozen with dry ice–ethanol. The frozen tissue could be stored at —20° for a few days. In the case of ascites tumors, the animals were killed by cervical fracture and the ascitic fluid was drained through an abdominal incision into tubes surrounded by ice. The cells were sedimented by centrifugation at 1,000 × *g* for 7 min. The packed cells were extracted with cold 0.2 *N* PCA. 1 *N* PCA was added to the supernatant fluid to give a final concentration of 0.2 *N*. Tumors* used in this experiment were ascites hepatoma 7974[5] (rat), sarcoma 180 (mouse), and Ehrlich tumor (mouse).

Thymidine and other chemicals were obtained commercially.

DNA was prepared from bovine spleen by the detergent method[6]. The fibrous product containing 8.5 % of phosphorus was contaminated by less than 2 % of RNA.

Enzymic casein hydrolysate. This was prepared by digestion of casein (Nutritional Biochemicals) with papain. An amount of concentrated hydrolysate containing 76 mg nitrogen (equivalent to 500 mg protein) was added to each 100 ml of the basal medium.

Snake venom. Vacuum-dried powder of venom of *Trimeresurus flavovidridus* or *Agistrodon blomhoffi* was used. Sometimes the recovery in microbioassay of DNA was found incomplete owing to the sample of venom used. Therefore such samples of venom as gave satisfactory recovery of DNA were selected for subsequent use.

Microbioassay. Deoxynucleosidic compounds were assayed microbiologically with *Lactobacillus acidophilus* R-26. The method was essentially the same as that described by HOFF-JØRGENSEN[2,3] except for the following modifications: uracil (2 mg/ml) and pyridoxine (100 μg/ml) were added to purine-pyrimidine and vitamin solutions respectively, and the concentrations of DL-tryptophan, L-cysteine and thioglycolic acid were doubled.

The overall procedure for the determination of deoxynucleosidic compounds, with or without snake venom digestion, is described in RESULTS AND DISCUSSION.

Determination of DNA. DNA contents of eggs and embryos were determined microbiologically, and those in rat tissues chemically[7].

RESULTS AND DISCUSSION

Effect of snake venom digestion

First, the acid-soluble extracts of eggs and embryos were analysed for their deoxynucleosidic compounds by the original method of HOFF-JØRGENSEN. As long as the original procedure was used, deoxynucleosidic material was scarcely detectable in these extracts.

By chance, it was found that digestion of the extracts with snake venom enzyme before microbioassay made the extracts highly active in supporting the growth of the test organism (Fig. 1).

* Tumor samples were kindly provided by Dr. K. KAZIWARA of Research Laboratories, Takeda Pharmaceutical Industries, Ltd.

There are three possible ways of interpreting this activating effect of the venom digestion. Firstly, the venom might activate a trace amount of DNA present in the acid-soluble extracts. But this was excluded by the following facts, indicating the absence of any detectable amount of DNA in the extracts: pancreatic deoxyribonuclease, which is known to activate DNA microbiologically[2,3], could not replace the venom in activating the extracts, and the active principle of the extracts was found to be completely dialyzable through cellophane membrane, whereas DNA is non-dialyzable.

The second possibility, that the venom digestion might remove an inhibitor, is highly unlikely because the addition of the acid-soluble extracts to a thymidine solution did not inhibit the recovery of the latter in the microbioassay. If there were any inhibitor, the recovery of thymidine should be depressed.

Thus we were led to accept the third possibility that there exists a new group of deoxynucleosidic compounds which are, in fact, activated by venom treatment and therefore not detectable by the usual microbiological method.

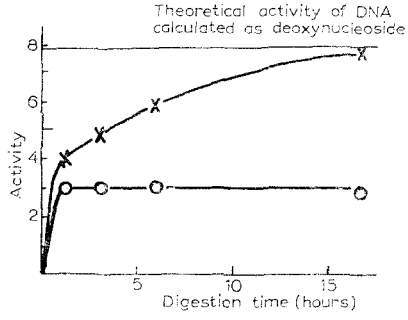

Fig. 1. Activation of acid-soluble deoxynucleosidic compounds of sea urchin eggs (O—O) and DNA (×—×) by snake venom digestion. Activity expressed as mμmole thymidine/ml digestion mixture. Conditions for digestion: snake venom, 250 μg/ml; $MgSO_4$, 0.01 *M*; potassium maleate, 0.06 *M*, pH 6.8; incubated at 37°.

We tentatively designated this group of deoxynucleosidic compounds as "masked" deoxynucleosidic compounds[4]. It is evident that the "masked" deoxynucleosidic compounds are neither simple deoxynucleosides, deoxynucleotides, nor products obtained by degradation of DNA with deoxyribonuclease, because the latter compounds are all active toward *L. acidophilus* without any pretreatment.

Analytical procedure with special reference to "masked" deoxynucleosidic compounds

Before attempting to clarify the chemical nature and biological significance of "masked" deoxynucleosidic compounds, it seemed necessary to establish a routine analytical procedure.

Tissues (dry defatted powder or fresh tissues weighing 10-100 mg or 50-500 mg, respectively) were extracted three times or more with 2–4 ml portions of cold 0.2 *N* PCA, using a glass homogenizer. The combined extracts were neutralized with 2 *N* KOH, kept overnight in a refrigerator and centrifuged to remove potassium perchlorate. The supernatant was diluted or concentrated to a given volume (usually 10 ml).

An aliquot of less than 4.0 ml was taken from this neutralized extract. To it was

added 1.0 ml of maleate–magnesium sulphate–venom solution of pH 6.8, containing 0.3 mmole potassium maleate, 0.05 mmole magnesium sulfate, and 2.0 mg snake venom, and the mixture was diluted to 5.0 ml with water and incubated for 3–16 h at 37° (under a layer of toluene or hexane). The digesting reaction was stopped by immersing a glass tube containing the reaction mixture in a boiling water bath for 5 minutes. The venom-treated acid-soluble fraction so obtained was referred to as ASt.

Another aliquot from the neutralized PCA extracts was treated in the same way except that snake venom was omitted from the reaction mixture and the resulting solution was referred to as ASu.

The residue from the cold PCA extraction was washed twice with cold ethanol and once with ether, dried, incubated with 2–5 ml 0.5 *N* KOH overnight at 37°, neutralized with 0.5 *N* maleic acid, and diluted to a given volume. The rest of the treatment of this acid-insoluble fraction was the same as that of acid-soluble fraction, and two separate aliquots served for making two samples, AIt and AIu, which were incubated with and without snake venom, respectively.

The four samples, ASu, ASt, AIu, and AIt, thus obtained, were assayed for their deoxynucleosidic growth activity according to HOFF-JØRGENSEN. Thymidine was used as an assay standard and all results were expressed as mμmoles thymidine equivalent.

As is evident from the above procedure, ASu represents the amount of simple deoxynucleosides and deoxynucleotides, and the difference, ASt minus ASu, represents that of "masked" deoxynucleosidic compounds. The difference, AIt minus AIu, on the other hand, represents the amount of DNA. In our experience, AIu was always microbiologically inactive, so that the analysis of AIu could usually be omitted.

Amount of simple and "masked" deoxynucleosidic compounds in eggs and embryos of sea urchin and tissues of the rat and mouse

Eggs and embryos of the sea urchin, the liver, kidney, spleen and tumors of the rat, and tumors of the mouse were analysed for their deoxynucleosidic compounds by our modified procedure.

Analytical results in sea urchin eggs and embryos at various developmental stages are summarized in Table I and those in rat and mouse tissues in Tables II and III.

Several interesting points are seen from these tables. First, as already mentioned, in eggs and embryos almost all of the acid-soluble deoxynucleosidic compounds are in the "masked" form and scarcely any simple deoxynucleosides or deoxynucleotides are found. This is in sharp contrast with the situation in adult rat tissues, where a relatively large portion of the acid-soluble deoxynucleosidic compounds consists of simple deoxynucleosides and/or deoxynucleotides. Secondly, the amount of "masked" deoxynucleosidic compounds remains almost constant during the early cleavage but shows a marked decrease during the later cleavage stage, when the rate of DNA synthesis and cell division gradually decreases.

Finally, it will be seen that the ratio of the acid-soluble "masked" deoxynucleosidic compounds to DNA is the highest in eggs before or immediately after fertilization and becomes lower with the progress of development, whereas the ratio is very low in the adult mammalian tissues.

All these facts suggest that the "masked" deoxynucleosidic compounds participate actively in DNA synthesis or some other biosynthetic reactions taking place in

TABLE I

DEOXYNUCLEOSIDIC COMPOUNDS CONTENT OF SEA URCHIN EGGS AND EMBRYOS

Material	*ASu**	*ASt**	*"Masked"* deoxynucleosidic compounds (ASt–ASu)*	*DNA* (Ait)*	*"Masked"*** deoxynucleosidic compounds / DNA*
Unfertilized eggs:					
No. 1	4.0	75	71	14	5.1
2	6.1	69	63	40	1.6
3	8.3	112	104	46	2.3
4	5.0	87	82	35	2.3
5	5.0	86	81	34	2.4
6	5.0	80	75	30	2.5
Embryos:					
stage 1 cell	—**	78	78	12	6.5
1 cell	—**	106	106	16	6.6
1 cell	—**	134	134	17	7.9
2 cells	—**	104	104	29	3.6
4 cells	—**	103	103	43	2.4
8 cells	—**	117	117	111	1.1
8 cells	—**	126	126	108	1.2
16 cells	—**	116	116	140	0.82
early blastula	2.0	46	44	1,350	0.033
early blastula	1.0	35	34	1,320	0.026
hatching blastula	—**	18	18	1,660	0.011

* Expressed as mμmole thymidine equivalent/100 mg dry weight. Since no appreciable change of dry weight per egg or embryo is observed during the early developmental stage studied, the values calculated on the basis of dry weight are roughly proportional to the amounts per egg or embryo.

** Trace.

*** The ratio of values of column 4 to those of column 5.

TABLE II

DEOXYNUCLEOSIDIC COMPOUNDS CONTENT OF RAT TISSUES

Material	*ASu**	*ASt**	*"Masked" deoxynucleosidic compounds (ASt–ASu)**	*DNA***	*"Masked"*** deoxynucleosidic compounds / DNA*
Liver: No. 1	42.7	92.3	49.6	8,710	0.0057
2	35.7	62.4	26.7	8,560	0.0031
3	25.9	51.7	25.7	8,880	0.0028
4	26.6	90.0	64.1	7,800	0.0082
5	37.8	68.4	30.6	8,070	0.0038
Spleen: No. 1	93.1	156	42.9	37,200	0.0012
2	96.7	143	46.3	39,500	0.0012
3	60.6	114	53.4	38,000	0.0014
4	100	158	58.0	41,500	0.0014
5	87.3	113	25.7	29,500	0.0009
Kidney: No. 1	53.3	53.0	0	12,400	0.0
2	48.6	60.6	12.0	12,800	0.0009
3	26.7	40.0	13.3	12,100	0.0012
4	33.5	33.5	0	12,500	0.0
5	51.5	80.0	28.5	12,100	0.0024

* Expressed as mμmole thymidine equivalent/1 g wet weight.

** Determined chemically and expressed as mμmole phosphorus/1 g wet weight.

*** See the footnote *** of Table I.

TABLE III

DEOXYNUCLEOSIDIC COMPOUNDS CONTENT OF TUMORS OF THE RAT AND MOUSE

Material	*ASu**	*ASt**	*"Masked" deoxynucleosidic compounds (ASt–ASu)**	*DNA***	*"Masked"§ deoxynucleosidic compounds / DNA*
Rat – Hepatoma 7974					
Solid					
No. 1	156	187	31	6,940	0.0045
2	106	150	44	6,580	0.0077
3	150	164	14	5,830	0.0024
Ascites cells					
No. 1	41	56	15	2,600	0.0058
2	53	62	9	—	—
Ascites fluid**					
No. 1	255	376	121	0	—
2	288	528	240	0	—
Mouse – Sarcoma 180					
No. 1	15	71	52	4,200	0.0124
2	26	71	45	7,570	0.0059
Ehrlich ascites					
cells	6	6	0	2,600	0
fluid**	8	36	24	0	—

* Expressed as mμmole thymidine equivalent/1 g wet weight or ml fluid.
** Determined chemically and expressed as mμmole phosphorus/1 g wet weight.
*** Supernatant fluid obtained by removing the cells by centrifugation.
§ See the footnote *** of Table I.

early embryos during cleavage stage. In this connection, it may be worth noting that during the cleavage stage no great change in the net amount of protein[8] or RNA[9] was found, while DNA shows a remarkable increase.

Data in Table II indicate that the presence of "masked" deoxynucleosidic compounds is not restricted to eggs and embryos but rather widely distributed in adult mammalian tissues including tumors, though their relative amounts compared with simple deoxynucleosidic compounds or DNA in the latter tissues are comparatively small.

Acid-soluble deoxynucleotides of "masked" form were also found in microorganisms and evidence has been presented indicating their active participation in DNA synthesis[11,12,13].

Recently, ROTHERHAM AND SCHNEIDER[15] have reported the presence of "digestible" deoxynucleotides, which are essentially the same as our "masked" deoxynucleosidic compounds, and have come to a conclusion similar to ours about their biological significance.

"Masked" deoxynucleosidic compounds of sea urchin eggs were completely adsorbed on Dowex-1-formate resin from the neutral solution, this indicates that they are acidic and perhaps the derivatives of deoxynucleotides. Further studies on their chemical nature led us to the discovery of a new conjugated derivative of deoxycytidylic acid, deoxycytidine diphosphate choline[14]. Details of these studies

and the chemical characteristics of the "masked" deoxynucleosidic compounds will be described in a subsequent paper[16].

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