On the Binding of Gelatine to Modified Serum Proteins Through Formaldehyde in an Experimental Blood Volume Expander

The preparation, in our Institute, of an experimental blood volume expander from bovine serum includes heat denaturation, formolation and an oxidation of serum proteins in the presence of a partially degraded gelatine (PDG)[1]. In the present study we tried to get information whether, and to what extent, the formation of intermolecular methylene links[2–4] takes place between serum proteins and PDG under the given conditions.

The experimental approach consisted in the separation and determination of bound and free PDG in the following standard samples. Sample A: a mixture of 4 volumes of bovine serum (5% protein) and of 1 volume of 4% PDG was heated at 100°C for 1 h at pH 8.5 in the presence of 0.13% formaldehyde. Sample B was prepared by oxidizing sample A by 1% hydrogen peroxide at 90–95°C for 10 min[1]. Sample C: to 4 Vol of 5% bovine serum formolated previously without gelatine in the same way as sample A, 1 Vol of 4% PDG was added afterwards at laboratory temperature. Sample D was a simple mixture of 4 Vol of 5% serum and 1 Vol of 4% PDG.

Gel filtration on Sephadex G-200 was used to separate the free low-molecular PDG (consisting of particles of m.w. 10,000–15,000)[5] from the main high-molecular fraction of the modified serum (with particles of an average m.w. 100,000–150,000)[5], to which a part of PDG was assumed to be bound through formaldehyde. The amount of bound PDG was then estimated by comparing the contents of hydroxyproline[6] in each sample before the gel filtration (this value was taken as 100%) and in the separated high-molecular fraction of the corresponding sample. Thus the samples A, B, C, and D contained bound PDG in ratios of 60%, 45%, 35%, and 0% respectively. These results, even though only semi-quantitative, still indicated a significant binding of PDG to modified serum proteins.

More accurate quantitative results, however, were achieved by another method[7] based on the precipitation of serum proteins together with bound PDG by 5% trichloroacetic acid. Free PDG which remained in solution during this operation was then determined turbidimetrically or densitometrically after reaction with tannin at pH 4.9. The content of free and bound PDG in the samples tested was expressed in %, taking sample D as having 100% PDG free, i.e. 0% of PDG bound to serum proteins. The results are presented in the Table. The standard deviations of the mean were calculated for $P = 0.05$.

The results shown in the Table confirm the existence of the intermolecular binding mentioned above, and they can be taken as an indirect proof of the formation of methylene links between the other protein molecules of blood volume expanders of this kind, as had been assumed. On oxidation (sample B) a part of bound PDG was released, probably because of the rupture of some bonds under the formation of formic acid[2,8].

Binding of gelatine to serum proteins in different samples

Sample	% of bound gelatine
A (serum + gelatine, formolated)	34.9 ± 3.1
B (sample A – oxidized)	18.8 ± 2.6
C (serum formolated, gelatine added afterwards)	5.5 ± 3.8
D (serum + gelatine)	0

Zusammenfassung. Beim Formolieren und Erhitzen einer Mischung von Rinderserum und teilweise abgebauter Gelatine wird ein Teil der Gelatine kovaliert an die Moleküle des bei diesem Prozess modifizierten Serums gebunden. Die Trennung der noch freien von der an die Serumproteine gebundenen Gelatine konnte durch Gelfiltration mit Sephadex G 200 und durch fraktionierte Fällung mit Trichloressigsäure erreicht werden.

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A Study of DNA Synthesis in Sea Urchin Hybrids by the Incorporation of H^3-Thymidine

In our previous studies[1,2] we have followed the incorporation of C^{14}-adenine into RNA during the development of both normal and hybrid sea urchin embryos. The present paper deals with a similar study on the synthesis of DNA by using H^3-thymidine as the precursor.

The hybrids studied were *Paracentrotus* ♀ × *Arbacia* ♂ (PA) and *Paracentrotus* ♀ × *Sphaerechinus* ♂ (PS). Both combinations die similarly at the early gastrula stage but are quite different in their cytological behaviour[3–5]. It would be of interest to know if and how they differ in their ability to take up labelled precursors into DNA. Tritiated thymidine has been shown to be incorporated in the nuclei of amphibian hybrids[6,7]. Analyses of DNA

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synthesis, by using tritium-labelled thymidine in sea urchin eggs and during normal development, have been reported by a number of previous authors[8-12]. After we had finished the present experiments in the spring of 1964, it became known to us that an autoradiographic investigation on the incorporation of various labelled precursors into DNA, RNA and proteins of the hybrid combination PA had been carried out by FICQ and BRACHET[13]. The results of these two authors will be compared with our own in later sections.

For PA the earliest stage studied by us was the newly hatched blastula aged about 17 h, because at this time the swimming blastulae can easily be separated from the unfertilized eggs. For PS earlier stages have to be considered, as the majority of S-chromosomes are already eliminated during the first cleavages[4,5]. With some minor modifications hybridization was done according to procedures given by HAGSTRÖM[14]. For PA eggs of *Paracentrotus* were pretreated for 10–15 min with sea water plus NaOH (pH 9.3), and sperms of *Arbacia* added in the presence of egg extract of the latter species (see ELSTER[15]). For PS the eggs were treated for 10–15 min with trypsin (Merck) before being inseminated with sperms of *Sphaerechinus* mixed with 0.5% glycine. The culturing temperature was 18°C.

The radioactivity of DNA was estimated as follows: embryos of desired stages were incubated for 2 h at room temperature (20–22°C) in sea water containing H^3-thymidine (final concentration 4.44 μc/ml). After washing in sea water containing unlabelled thymidine, alcohol and ether, DNA was extracted with perchloric acid according to the method of SCOTT et al.[16]. The extract was then neutralized, thoroughly dried, and its radioactivity estimated by a windowless gas flow counter. For PP, PA, PS and SS 100 embryos and for AA 200 embryos were used for each analysis.

In recent years, parallel to the isotopic studies, we have also counted the numbers of nuclei of various developmental stages in squashed preparations, employing at first the technique described in CHEN and BALTZER[2] and then the Gomori staining. Whenever DNA is dealt with, it is of importance to know the mitotic activity as indicated by the increase of nuclei. In evaluating the results of the nuclear counts, it must be remembered that AA and especially SS develop much more slowly than PP. When PP has reached the stage of pluteus with four arms (Pl. 2, see Figures in WHITELEY and BALTZER[3]), AA stands still between the prism and the early pluteus (Pl. 1), and SS even remains far behind at the late gastrula stage (GaJ 1/1).

Our overall data of radioactivity are summarized in the Table. These include, for both normal and hybrid embryos, at least four different stages, and each value represents the average determined from two parallel samples.

(1) *The hybrid PA.* In agreement with our previous findings on total DNA[17], PP and AA differ quantitatively in their rates of thymidine incorporation. The values of PP are at first four times and in later stages two to three times higher than those of AA. A similar difference has been found for the numbers of nuclei between these two species[2]. At the age of 17–29 h the incorporation rate of the hybrid PA is about intermediate between the parental species and corresponds to about half of PP. However, at later stages (35–47 h) it drops far below the normal range. Likewise there is a distinct reduction in its nuclear numbers: at 47–48 h of age PP, which has developed to pluteus in the meantime, has 1429 nuclei, whereas PA, which stopped development during gastrulation, has only 641 nuclei. The corresponding value of AA is 685 (see CHEN and BALTZER[2], p. 237; owing to individual variations the nuclear numbers given here are only approximate values). Thus there is a reasonable agreement between the rate of incorporating labelled thymidine into DNA and the numbers of nuclei.

(2) *The hybrid PS.* As already mentioned, the majority of the paternal chromosomes are eliminated at the first cleavages[4,5]. In the blastulae aged 8 h, the blastocoel is still empty, but the germ wall containing abnormal chromatin-vesicles is less regular and thicker in PS than

Incorporation of H^3-thymidine into DNA at different developmental stages of normal and hybrid sea urchin embryos

Developmental stage	Age in h after fertilization (18°C)	No. of determinations	Radioactivity (cpm/100 embryos)
PP (6 series)			
Morula	4 h 30– 5 h 30	4	122.4
MyBl	14 h 40–17 h 20	10	120.5
Ga J 1/2-3/4	21 h 30–22 h 05	4	112.9
Ga J 4/5-Pri	24 h 10–29 h 45	10	155.1
Pl-1	35 h 35–41 h 25	8	115.4
Pl-2	45 h 00–48 h 20	8	99.4
AA (3 series)			
MyBl	17 h 00–17 h 20	6	30.0
Ga J 1/2-2/3	24 h 10–28 h 00	6	39.6
Ga J 2/3-Pri	35 h 55–41 h 25	4	49.8
Pl-1 (2)	45 h 00–52 h 25	8	52.7
SS (2 series)			
Morula	6 h 00	2	60.4
Bl (before hatching)	14 h 40	2	66.8
Bl (after hatching)	21 h 30–22 h 05	4	75.9
MyBl	27 h 50	2	72.5
Ga 1/3-2/3	36 h 00	2	74.5
Ga J 4/5-Pri	45 h 00	2	92.1
PA (3 series)			
MyBl	17 h 00–17 h 40	6	61.3
Ga J 0-1/2	24 h 15–29 h 45	6	62.2
Ga J 1/4-3/4	35 h 40–42 h 05	6	23.2
Ga J 1/4-3/4	45 h 05–47 h 10	6	26.4
PS (3 series)			
Morula (chrom. elim.)	4 h 30– 6 h 05	4	60.1
Stereoblastula	14 h 40–16 h 00	4	46.8
Stereoblastula/GaJO	21 h 30–22 h 05	4	40.5
Stereoblastula/GaJO	27 h 50	2	22.3
Stereoblastula/GaJO	38 h 55	2	15.6
Stereoblastula/GaJO	45 h 00	2	18.6

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in PP (see Figure 4 in BALTZER and CHEN[5]). Hybrid embryos aged 15 h are stereoblastulae, filled with degenerating materials in their cavity. A part of these embryos attempts to gastrulate, though later than PP, but does not develop further. The early abnormality resulting from the elimination process is distinctly reflected in the values of thymidine uptake. Although the values of hybrid embryos at the cleavage stages ($4^1/_2$–6 h) still amount to approximately half of PP, they very soon drop behind and are much lower than those of both parental species at 15 h already: PP about 120, SS 67, and PS 47. Finally at 39–48 h DNA synthesis in the hybrid drops to only about one seventh of that in the maternal controls.

Again there is a certain parallelism between the rate of thymidine incorporation and the nuclear numbers. At 18–20 h there are about 400 nuclei in PS, compared to about 600 in PP and 400 in SS. But the value rises to 517 and 670 in the PS hybrid aged 41–48 h, whereas within the same period it increases to 956 and 1400 in PP and to 669 and 692 in SS (compare CHEN and BALTZER[2], p. 237, and some recent counts).

From their autoradiographic experiments, FICQ and BRACHET[13] concluded that the incorporation of H^3-thymidine into DNA is higher in AA than in PP. A direct comparison between their study and the present one is not possible because, in addition to differences in the techniques used, they investigated mainly the early development while more data are available to us for later stages. Besides the rate of development, which is slower in AA than in PP, other factors like the pool size and the cytoplasmic uptake of the precursor might account for such a discrepancy.

The data presented by FICQ and BRACHET[13] further indicate a considerably higher incorporation of thymidine in PA than in PP for the stages investigated by them. On the other hand, we found a much lower uptake of this labelled precursor in PA than in PP, at least from the mesenchyme blastula stage onwards. Since they disclosed that DNA synthesized by the hybrid is abnormally unstable, it seems possible that the low values of PA determined by us are partly due to loss of some labelled DNA by the extraction procedure.

Another point of interest is that their autoradiographs suggest the elimination of chromatin in the cytoplasm of the PA hybrid. However, in agreement with our observation, no chromatin elimination could be detected by the Feulgen staining. This phenomenon certainly deserves a closer examination.

According to evidence available, protein formation takes place through the template mechanism of RNA which is in turn DNA-dependent. It would be of interest to know to what extent the inhibition of DNA synthesis is directly related to the lethality of the hybrids. During the first 17 h of development, almost all PA hybrids develop normally and do not differ from the PP controls. Thereafter both types of embryos are characterized by an indentation of the vegetative part, indicating the beginning of gastrulation. It is at this critical stage that the development of PA becomes retarded. Even though up to this time PA develops as normally as the maternal controls, its DNA synthesis, as indicated by the incorporation of H^3-thymidine, is already reduced to about half of PP. The real role of DNA of *Arbacia* in the present hybrid combination is unknown. According to MOORE[18], the abnormal development could be a consequence of inexact copies of the genetic material. But, as suggested by BRACHET[19] and BRACHET et al.[20], the possibility that it affects indirectly the embryogenesis through an abnormal synthesis or utilization of RNA is not excluded. Unfortunately, we have no data for still earlier stages. A comparison between this hybrid and the merogonic combination (P)A would be desirable[21].

Zusammenfassung. (1) Es wird die DNS-Synthese der letalen Seeigelbastarde *Paracentrotus* ♀ × *Arbacia* ♂ (PA) und *Paracentrotus* ♀ × *Sphaerechinus* ♂ (PS) mit H^3-Thymidin untersucht. (2) Dem verschiedenen, der Letalität vorausgehenden Entwicklungstypus (PA ohne, PS mit Chromosomenelimination) entspricht ein verschiedener Verlauf der Hemmung der DNS-Synthese. Parallel dazu wird die Vermehrung in der Anzahl der Kerne verglichen.

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Agglutinating Antibrain Antibodies in Dogs with Experimental Allergic Encephalomyelitis

Although experimental allergic encephalomyelitis (EAE) has been induced in various animals, it has not always been possible to reveal circulating antibrain antibodies in these animals[1,2]. Antibrain antibodies have been detected in the sera of dogs and monkeys with EAE, without a definite correlation between the occurrence of circulating antibodies and the occurrence of the disease being ascertained[3,4]. Animals have been found which had circulating antibrain antibodies without clinical signs of EAE, and animals that displayed severe signs of EAE without having antibrain antibodies in their sera[1].

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