NEW HORIZONS IN BIOLOGICAL CHEMISTRY

Edited by
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NEW HORIZONS IN BIOLOGICAL CHEMISTRY

The Proceedings of the Symposium Held in Honor of
Professor Kunio Yagi on the Occasion of
the 60th Birthday Anniversary under the Auspices of
the Japanese Biochemical Society

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PREFACE

On behalf of the organizing committee, it is my great pleasure to address all the speakers and participants on the occasion of the 60th birthday anniversary of Prof. Kunio Yagi. We organized this meeting under the auspices of the Japanese Biochemical Society, in honor of Prof. Kunio Yagi as well as to communicate findings among several different fields of biochemistry.

The principle of this symposium is to invite approximately 20 outstanding researchers in biochemistry from all over the world, including Prof. Yagi's intimate friends, and to ask them to give us plenary lectures. Thus it will cover fields from genetic information such as DNA to biomedical chemistry such as calcium transport, neuromodulators or muscle contraction. Biochemistry is, in a sense, a rather young and rapidly developing science. We are now in a situation similar to that of the great navigation age of the 16th Century; we still do not know where the new continents lie and what new horizons there are in biochemistry. So the aim of this symposium is not the usual one of obtaining a consensus in a small restricted field. We believe this symposium will promote the exchange of new scientific ideas and knowledge among participants from a wide range of biochemical fields and promote mutual friendship between them.

The organizing committee asked all speakers to give a lecture on their recent achievements with some historical background and their outlook on new horizons in biochemistry.

We will listen to the speakers' own goals in biochemistry, perhaps 23 different ones, just as Christopher Columbus' goal was India while Captain Cook sought Australia. We expect this will be a very enjoyable event. The organizing committee expresses hearty thanks to all speakers and to the chairmen who gathered in Nagoya from all points of the globe, and to all participants young and old. We hope everyone enjoys the leactures and strengthens friendly ties. Finally, we would like to express our gratitude to the Japanese Biochemical Society for their help in organizing this symposium.

November 1st, 1979 Takayuki OZAWA

CONTENTS

EXPERIMENTAL APPROACH TO CHEMICAL EVOLUTION IN THE PRIMEVAL SEA AND A HYPOTHESIS ON THE ORIGIN OF THE GENETIC CODE

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EXPERIMENTS ON CHEMICAL EVOLUTION

For several years we have been engaged in the experimental approach to chemical evolution in the primeval sea.[1-5] This plan is based upon the following assumptions:

1. The origin of life, the chemical evolution just preceding it, and early biological evolution occurred in the primeval sea under an anoxygenic atmosphere.[6,7]

2. Chemical evolution in the primeval sea was accelerated by transition metals. Six transition elements (Mo, Fe, Zn, Mn, Co, Cu) essential to most prokaryotes[1] must be taken into consideration.

We have thus set up a modified sea medium enriched with the following transition metals: HPO_4^{2-}, SO_4^{2-}, Mg^{2+}, Ca^{2+}, each at 0.01 M, Na^+ 0.015 M, K^+ 0.05 M, Cl^- 0.07 M, NO_3^- 0.0005 M, Zn^{2+}, MoO_4^{2-}, Fe^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, each at 0.0001 M, and pH was adjusted to 5.5 following the addition of starting materials.

The media with appropriate starting materials were kept at 105° for several days or even for several weeks under anoxygenic atmosphere. A temperature that would accelerate the reaction and exclude any microbial contamination was chosen.

Initially we chose a one-carbon compound, formaldehyde, and a one-nitrogen compound, hydroxylamine, as the starting materials (CH_2O 0.30 M and NH_2OH 0.05 M or CH_2O 0.5 M and NH_2OH 0.5 M). A series of amino acids were produced in a period of several days. These were tentatively identified by the retention time in the automatic amino acid analysis (Gly, Ala, Ser, Thr, Ile, Asp, Glu, Val, Leu, Arg, Lys, Pro, His, *etc.*). Among these, the main protein amino acids also confirmed were Gly, Ala, Ser, Asp, and Glu. Serine and aspartic acid were produced from glycine and formaldehyde in a higher yield.

It should be pointed out that not only free amino acids but also amino acid polymers which give rise to amino acids by acid hydrolysis were produced. It is rather remarkable that amino acid polymers—degree of polymerization was 2–7—were produced in an aqueous medium.[1] The simplest among these was glycylglycine.[2] The mechanism of polymer formation remains unknown. However, we believe that the polymers were produced through a multimolecular, multicatalytic system. It is similar to the formation of ATP, by alcoholic fermentation in an aqueous medium: $C_6H_{12}O_6+2ADP+2P_i \longrightarrow 2C_2H_5OH+2CO_2+2ATP+2H_2O$. This reaction comes about by a multimolecular, multicatalytic system of alcoholic fermentation. I hold the view that chemical evolution is the establishment and development of multimolecular, multicatalytic systems, which resulted in the origin of life characterized by organized structures and remarkable synthetic activities including self-replication. This is the reason why we began the study of chemical evolution using modified sea water enriched with six transition element ions, which probably accel-

erated chemical evolution as complex catalysts. I find a germ of the multimolecular, multicatalytic system in our experiments.

In order to study further the formation of amino acid polymers, we have used amino acid mixtures as starting materials. Polymers with more than about 2,000 daltons were produced.[8] Extensive studies have been carried out with an amino acid mixture consisting of glycine and, acidic, basic, and aromatic amino acids (Gly 0.05 M, L-Glu, L-Asp, L-Lys, L-Arg, L-His, L-Trp, L-Phe, L-Tyr, each 5 mM).[5] We have observed the formation of polymerized products with complex structures. In the course of this study we were surprised by an unexpected finding: well-organized particles, shown in Fig. 1 separated out from the medium after several weeks. We designated these particles marigranules, since such particles were probably produced in the primeval sea. The properties of the marigranules are as follows: melting point $>300°$, if any. Elemental analysis: C 58.22, H 3.76, N 14.23, Ash 7.62%, IR_{max}^{KBr} 3,560–2,000, 1,710, 1,680–1,610*, 1,550*, 1,510, 1,450, 1,380, 1,260, 1,160, 1,130, 850, 750 cm^{-1}. The existence of peptide bonds is suggested*. These granules consist of two parts: a surface layer soluble in ethanol and an interior part soluble in 1 N KOH. The solubilized marigranules were hydrolyzed by elastase and more than 30 % of the nitrogen content was liberated in the form of an amino group.

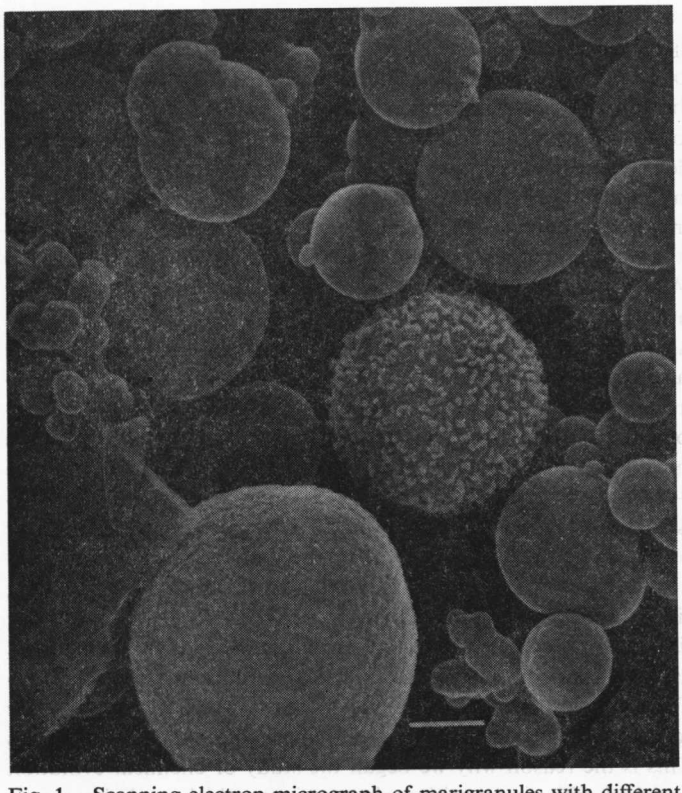

Fig. 1. Scanning electron micrograph of marigranules with different surface structures (H. Yanagawa). Bar, 1 μm. Cited from ref. 5.

The marigranules join the group of protocell-like structures such as the well-known coacervate droplets of Oparin[9] and the proteinoid microspheres of Fox.[10]

HYPOTHESIS ON THE ORIGIN OF THE GENETIC CODE

In the course of study on the formation of amino acids from formaldehyde and hydroxylamine I discovered a correlation between the first and second letters of the genetic codons and the number of carbon atoms in the carbon skeleton of amino acids.[11]

As mentioned above, the predominant protein amino acids produced from formaldehyde and hydroxylamine were glycine, alanine, serine, aspartic acid, and glutamic acid. Guanine is found in the two-letter genetic codons of these amino acids as shown below:

$$\begin{matrix} CH_2O \\ NH_2OH \end{matrix} \longrightarrow \underset{\text{Gly(GG)}}{C_2} \xrightarrow{+C_1} \underset{\begin{matrix}\text{Ala(GC)} \\ \text{Ser(AG)}\end{matrix}}{C_3} \xrightarrow{+C_1} \underset{\text{Asp(GA)}}{C_4} \xrightarrow{+C_1} \underset{\text{Glu(GA)}}{C_5}$$

In other words, G is found in the two-letter codons of amino acids which may be regarded as produced by the step-wise addition of carbon atoms to glycine in the course of chemical evolution in the primeval sea. It may be expressed as G: Gly$+n$C$_1$, where n is 0, 1, 2, and 3.

This finding led me to consider that other nucleotide bases, A, U, and C, might be expressed similarly, and this was found to be so.

A simple arithmetic correlation was found between the two-letter codons and the number of carbon atoms in the carbon skeleton of amino acids as shown in Table I. Here, the third letter in the codons was neglected. It is probably of little importance, if any, in the primitive code.[12] I should like to explain several typical examples among the two-letter codons of amino acids (Fig. 2). Leucine has two codons, UU and CU. Leucine may be regarded as being composed of two C_3 (U), and thus is UU, and is considered to have a C_6 skeleton (U) with a C_5 main chain (C) and a C_1 side chain, and CU. Phenylalanine may

TABLE I

THE GENETIC CODE AND THE CARBON SKELETON OF AMINO ACIDS

[G] means C_2(Gly)$+n$C$_1$, where n is 0, 1, 2, and 3; [A] means nC$_2$, where n is 2 and 3; [U] means nC$_3$, where n is 1, 2, and 3; [C] means C$_5$.
Several amino acids may be assigned to two groups: Cys UG, Asp GA, Arg CG, Ile AU, Leu CU.

[G]	C_2:	Gly GG
	C_2+C_1:	Ala GC, Ser AG, Cys UG
	C_2+2C_1:	Asp GA
	C_2+3C_1:	Val GU, Glu GA, Arg AG and CG
[A]	$2C_2$:	Asp GA, Asn AA, Thr AC, Met AU
	$3C_2$:	Ile AU, Lys AA
[U]	C_3:	Ser UC, Cys UG
	$2C_3$:	Leu CU and UU, Ile AU
	$3C_3$:	Phe UU, Tyr UA
[C]	C_5:	Gln CA, His CA, Arg CG, Pro CC, Leu (C_5+C_1 side chain) CU

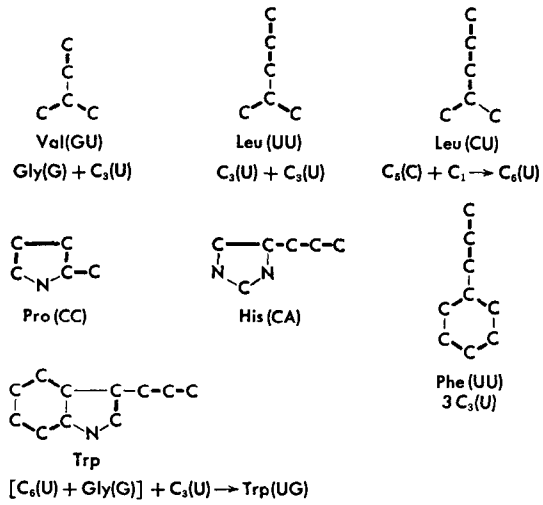

Fig. 2. Some examples of carbon skeletons.

be regarded as consisting of a C_6 ring (U) and a C_3 chain (U), then UU. Valine may be regarded as being composed of glycine (G) and a C_3 chain (U), then GU. Proline, glutamine, histidine, and arginine have quite different structures and properties, but they each have a C_5 chain, and their two-letter codons each contain C. Tryptophan is complicated. It is synthesized in extant organisms by the condensation of indole and serine (C_3). Indole may be regarded as consisting of a C_6 ring (U) and glycine (G), and then tryptophan has the two letter codon (UG).

I do not believe that the remarkable correlation could be established by chance alone, but must have some relation to the genesis of the genetic code.

The interaction among amino acids with their respective nucleotide bases was a prerequisite for establishing the genetic code. The interaction or complex formation of amino acids and nucleotide bases depends on the association constant (K_a) and the concentration of amino acids (A) and nucleotide bases (B):

$$[A \cdot B] = K_a[A][B]$$

The remarkable correlation among the genetic codons and the number of carbon atoms in the carbon skeleton of amino acids may be essentially a reflection of the concentration of coexisting amino acids and nucleotide bases in the primeval environments.

Based on this concept and taking into consideration the chemical and biochemical synthetic pathways of nucleotide bases and protein amino acids, I have been led to a working hypothesis for the interdependent genesis of nucleotide bases, protein amino acids, and primitive genetic codes.[13] This hypothesis differs from speculations so far presented (reviews to be consulted: refs. 14–17).

Amino acids can be classified into two groups, the Purine Group and Pyrimidine Group. Both groups can be further classified into two Subgroups, Guanine Subgroup or (Gly+

nC$_1$) Subgroup and Adenine Subgroup or nC$_2$ Subgroup, and Uracil Subgroup or C$_3$C$_6$C$_9$ Subgroup and Cytosine Subgroup or C$_5$-chain Subgroup, respectively, as follows:

Purine Group

Guanine Subgroup or (Gly+nC$_1$) Subgroup: Gly GG, Ala GC, Ser AG, (Cys UG), Asp GA, Glu GA, Arg AG, Val GU

Adenine Subgroup or nC$_2$ Subgroup: Asn AA, Thr AC, (Asp GA), Lys AA, Ile AU

Pyrimidine Group

Uracil Subgroup or C$_3$C$_6$C$_9$ Subgroup: Ser UC, Cys UG, Leu UU, CU, (Ile AU), Phe UU, Tyr UA

Cytosine Subgroup or C$_5$-chain Subgroup: Gln CA, His CA, Arg CG, Pro CC, (Leu CU)

Tryptophan and methionine with characteristic codons are not included in these groups. They were probably incorporated much later, in the course of early biological evolution.

The purine bases (guanine and adenine) and amino acids of the Purine Group may be regarded as being derived from C$_1$ compounds and N$_1$ compounds (including such compounds as HCN), which were abundant in the early primeval sea. Glycine was probably produced first, and from it Ala, Asp, *etc.* were produced by the step-wise addition of carbon atom. Probably in the relatively early course of chemical evolution, a C$_4$-amino acid precursor was synthesized through the condensation of a C$_2$ compound to glycine and this initiated the synthesis of amino acids of the Adenine or nC$_2$ Subgroup of the Purine Group.

As stated, G and A predominate in the codons of the amino acids of the Purine Group. This may be related to the similarity between the primitive synthetic pathways for these amino acids and the purine bases. As is well known, purine bases are synthesized in extant organisms from glycine and C$_1$ compounds. As Oró discovered, purine bases can easily be chemically synthesized from C$_1$, N$_1$ compounds such as HCN.[18] In any case, the synthesis of purine bases does not require any ready-made C$_3$-chain compounds. Thus, it is highly probable that these purine bases could have been synthesized from C$_1$ compounds and N$_1$ compounds and accumulated in the early primeval sea in higher concentrations, together with the amino acids of the Purine Group.

Pyrimidine bases and amino acids of the Pyrimidine Group may be regarded as being derived from compounds with C$_3$ or longer chains and N$_1$ compounds. The accumulation of such compounds in the primeval sea occurred either later than the predominant accumulation of C$_1$ and C$_2$ compounds or in a specific place where the concentration ratio C/N was much higher and such chain compounds were able to accumulate. At any rate, again there is a similarity between the synthetic pathways of these amino acids and that of the corresponding pyrimidine bases. Pyrimidine bases are generally synthesized from urea and C$_3$-chain compounds in organic chemistry. In their biosynthesis, the C$_3$-chain in pyrimidine derives from aspartic acid by the subsequent loss of CO$_2$. It is reasonable to assume that during chemical evolution, pyrimidines were synthesized from urea or some compound similar to guanidine and C$_3$-chain compounds as shown by Ferris *et al.*[19] and accumulated with amino acids of the Pyrimidine Group.

Thus, I suggest that the primitive genetic code was established through specific interaction between amino acids and their respective nucleotide bases. As mentioned above, this interaction was dependent not only upon the binding constants between amino acids and their respective nucleotide bases, but also upon the concentration of those in the primeval environments. Thus, depending upon the latter condition, the group of purine-rich

codons and the group of pyrimidine-rich codons were established. Of course, the two-letter codons were established depending upon both conditions (binding constant and concentration). Thus, some correlations are found between the codons and the structure and properties of corresponding amino acids.[14]

Different speculations have been presented on the origin of the genetic code. However, these speculations do not explain why serine has two quite different codons, UC and AG. This may be explained by the present hypothesis, as follows: serine was produced in the course of chemical evolution by two quite different means: by the amination of a C_3 compound (U subgroup) and by the addition of a C_1 compound (probably formaldehyde) to glycine (G subgroup).

In conclusion, based upon the remarkable correlation between the genetic codons and the number of carbon atoms in the carbon skeleton of amino acids, I suggest that the primitive genetic code was established by a specific interaction between amino acids and the respective nucleotide bases. This interaction was dependent upon their concentrations in the primeval environments and the binding constants between amino acids and their respective bases.

Finally, it should be pointed out that quite recently reports have appeared suggesting the direct interaction of amino acids with nucleotides of anticodons instead of codons.[20–22] If so, some difficulty is involved in explaining the cause of the correlation between the genetic code and the nature of the carbon skeletons of amino acids.

Still, I hope that the present working hypothesis, although far from complete, will provide a reliable basis for further studies on the origin of the genetic code.

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THE EXPRESSION OF MILK PROTEIN GENES

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The mammary gland provides an excellent eukaryotic system in which to study not only the fundamental mechanisms required for the regulation of specific gene expression, but also the cellular mechanisms peculiar to the post-translational modification and secretion of proteins.

Thus the mammary gland is a target for steriod and peptide hormones which interact to modulate milk protein gene expression,[1] but also as we emphasized in our lecture in Nagoya two years ago, milk proteins are synthesized *in vitro* as precursor polypeptides with peptide extensions at the NH_2-terminus. It is known that *in vivo* this 'signal' peptide plays a fundamental role in the intracellular segregation of secretory proteins, and it has now been established that the primary translation product of all the caseins and α-lactalbumin of every species studied, is synthesized as such a precursor.[2-8]

On this occasion, and more especially since the symposium is intended to stress the new opportunities for research in biochemistry, we wish to concentrate on the first area of study, namely gene expression. Clearly this is of considerable interest to molecular biologists and endocrinologists, but it is also of importance for two other reasons. Firstly that in the cow, milk proteins are an important source of a widely accepted food, and secondly because mammary tumours are the most common of all cancers occurring in women.

Many different mammals have been used experimentally to study the biosynthesis of milk proteins. Our own interest has centred on the guinea-pig, and we will be concerned in this review mainly with our work on this animal, and in women. However, various groups have used rats, mice, cows, goats, pigs, and rabbits.[9]

The milk proteins synthesized are in general terms very similar in all the mammals mentioned. They can be easily separated into two fractions, the caseins and the whey proteins. The latter constitute quite a variety of proteins but in quantitative terms only two proteins are important, α-lactalbumin which is found in the whey of all mammals, and β-lactoglobulin which is confined to ruminants. In this review we will be concerned, therefore, with the caseins and α-lactalbumin. The caseins are characterised by high levels of glutamic acid and proline, the absence of cysteine, and the presence of phosphate groups. Some are also glycosylated. These secondary modifications have complicated the characterisation of the *in vitro* biosynthesized caseins. α-Lactalbumin in the cow, guinea-pig, and rabbit is not glycosylated and has a mol. wt. of about 15,000. In the rat there appear to be two α-lactalbumins of apparent mol. wts. of 22,500 and 21,500 as determined by SDS-gel electrophoresis. Thus the rat α-lactalbumins are interestingly different from those of other species.[10]

In this review we will discuss several aspects of work currently in progress on the mammary gland, with particular emphasis on the regulation of milk protein gene expression.

ORGAN CULTURE

Early studies on the hormonal regulation of milk protein biosynthesis evolved around organ cultures.[11] This technique permitted studies on the action of individual hormones not possible in the intact animal. Using such systems Topper and Oka[12] established that maximal casein synthesis requires the presence of insulin, prolactin, and hydrocortisone. However Terry *et al.* showed that for mammary development in organ culture, aldosterone, oestrogen, and progesterone are also required.[13] Unfortunately such studies do not identify the intracellular site of action of these hormones, in particular prolactin (a peptide hormone), whose presence is a prerequisite for milk protein synthesis.

However, the use of organ culture, in conjunction with radiolabelled DNA hybridisation probes complementary to milk protein mRNA sequences, has revealed that prolactin acts both at the transcriptional and post-transcriptional levels. Thus, at present, evidence demonstrates that the action of prolactin is to increase the overall concentration of milk protein sequences.[14,15] In the rat Guyette *et al.*[16] have shown that this is achieved by a 2–4 fold increase in the rate of transcription of the casein genes, coupled with a 17–25 fold increase in the stability of milk protein mRNA sequences, when compared with other cellular mRNA sequences. The preferential effect of prolactin on the stability of milk protein mRNA sequences has also been reported in a rabbit mammary gland organ culture system by Houdebine *et al.*[17]

Our own attempts to study the effects of hormones on the regulation of guinea-pig milk protein gene expression, using either cells in culture or organ culture had for some time proved unsuccessful. However, recent work in our laboratory has demonstrated that provided medium from guinea-pig pituitary organ culture is used as a source of prolactin, then an increase in milk protein mRNA sequence is observed. The addition of purified rat or ovine prolactin did not produce the lactogenic response.

Although the use of organ culture, in conjunction with purified nucleic acid hybridisation probes, has provided considerable insight into the regulation of milk protein gene expression, it would be more useful to establish a mammary epithelial cell-line that responded to prolactin and other hormones in an identical manner to the organ culture system. This has proved difficult. The most hopeful approach seems to be that developed in the laboratory of Pitelka. Thus they have described the use of cultures of mid-pregnant mouse mammary epithelial cells on floating collagen membranes.[18] Such cultures respond to prolactin, insulin, and cortisol with the result that casein synthesis, as determined by radioimmunoassay, was increased markedly. They have now described the sustained growth of primary mouse mammary tumours and normal mammary epithelial cells embedded in collagen gels.[19] These cultures should prove useful in studies designed to determine the precise mode of action of prolactin. In this respect, it is interesting that evidence has accumulated which suggests that peptide hormones enter the cell[20,21] and even act directly at the nuclear level in an analogous manner to the proposed mechanism of action of steroid hormones.

TRANSCRIPTION AND ORGANISATION OF MILK PROTEIN GENES

The use of organ cultures in conjunction with nucleic acid hybridisation probes has provided insight into the hormones involved in milk protein gene expression, and to some extent their intracellular mode of action. However these studies do not provide information

relating to the number, size, or relative organisation of the milk protein genes. Neither do they provide information concerning the manner in which the genes are transcribed, or the requirement or otherwise for post-transcriptional processing events, resulting in the expression in the cytoplasm of active milk protein mRNA species.

However the application of techniques recently developed in other areas of molecular biology, combined with classical subfractionation of the lactating guinea-pig mammary gland, has provided information related to these questions.

For instance, whilst several different caseins may be detected in the milk of any particular species, the relationship between the different caseins had not been clarified until recently. Thus, there always seemed the possibility that the major caseins might be derived from a common giant precursor polypeptide. This in turn would be specifically processed into the individual lower mol. wt. caseins, subsequently identified in mammary secretions. Work using mRNA directed cell-free protein synthesizing systems clearly excludes such a possibility. We, for instance, have recently demonstrated *in vitro* that guinea-pig caseins and α-lactalbumin are all synthesized as individual polypeptides and do not result from the post-translational cleavage of a larger polypeptide.[4]

Similarly, characterisation of milk protein mRNA sequences in nuclei isolated from the lactating mammary gland, demonstrates that within the poly(A)-containing RNA

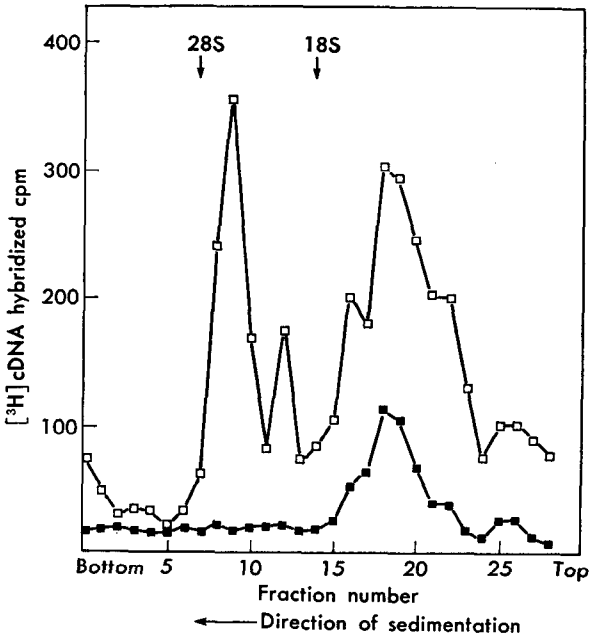

Fig. 1. Detection of a 25S nuclear poly(A)-containing RNA species in the lactating guinea-pig mammary gland which contains milk-protein mRNA sequences (from Bathurst *et al.*[22]). Nuclear (□) and post-nuclear (■) poly(A)-containing RNA sequences were fractionated on 5–20 % (w/v) sucrose gradients in the presence of 75 % (v/v) formamide. RNA species greater in size than 18S were separately pooled, and the fractionation procedure repeated. The distribution of milk protein sequences was then determined throughout the gradient using a ^{3}H-labelled cDNA hybridisation probe (see Craig *et al.*[3]).