Fudenberg, H. H. (1967) *Methods Immunol. Immunochem. 2,* 307.
Gerchman, L. L., & Ludlum, D. B. (1973) *Biochim. Biophys. Acta 208,* 310.
Gerchman, L. L., Dombrowski, J., & Ludlum, D. B. (1972) *Biochim. Biophys. Acta 272,* 672.
Goth, R., & Rajewsky, M. F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 639.
Halloran, J. J., & Parker, C. W. (1966) *J. Immunol. 96,* 379.
Kirtikar, D. M., & Goldthwait, D. A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 2022.
Kleihues, P., & Cooper, H. K. (1976) *Oncology 33,* 86.
Kleihues, P., & Margison, G. P. (1974) *J. Natl. Cancer Inst. 53,* 1839.
Lawley, P. D., & Shaw, S. A. (1972) *Biochem. J. 128,* 117.
Lawley, P. D., & Thatcher, C. J. (1970) *Biochem. J. 116,* 693.
Lawley, P. D., Orr, D. J., & Shaw, S. A. (1972) *Chem.-Biol. Interact. 4,* 431.
Levine, L., & Gjika, H. (1974) *Arch. Biochem. Biophys. 164,* 583.
Levine, L., Seaman, E., Hammerschlag, E., & Van Vunakis, H. (1966) *Science 153,* 1666.
Levine, L., Waalkes, T. P., & Stolbach, L. (1975) *J. Natl. Cancer Inst. 54,* 341.
Loveless, A. (1969) *Nature (London) 223,* 206.
Margison, G. P., & Kleihues, P. (1975) *Biochem. J. 148,* 521.
Minden, P., & Farr, R. S. (1967) in *Handbook of Experimental Immunology* (Weir, D. M., Ed.) p 463, F. A. Davis Co., Philadelphia, Pa.
Minden, P., Anthony, B. F., & Farr, R. S. (1969) *J. Immunol. 102,* 832.
Natali, P. G., & Tan, E. M. (1971) *Radiat. Res. 46,* 506.
Sawicki, D. L., Beiser, S. M., Srinivasan, D., & Srinivasan, P. R. (1976) *Arch. Biochem. Biophys. 176,* 457.
Singer, B. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 15,* 219.
Singer, B. (1976) *Nature (London) 264,* 333.
Sweetman, L., & Nyhan, W. L. (1968) *J. Chromatogr. 32,* 662.

Adenosine Incorporation by Unfertilized Mouse Ova: Adenylation of RNA and Adenosine Diphosphate Ribosylation of Protein†

R. J. Young* and K. Sweeney

ABSTRACT: Unfertilized mouse ova incorporated [^{3}H]adenosine into cold trichloroacetic acid insoluble material at a level which was highest 3–5 h after ovulation but which decreased sharply thereafter. Not all of the [^{3}H]adenosine-labeled material was released into the aqueous layer by chloroform–phenol extraction of labeled ova lysates. The labeled material in the aqueous layer was hydrolyzed by ribonucleases A and T_1 to [^{3}H]adenosine and a fragment which was converted by 0.3 M KOH to 3′-[^{3}H]AMP and [^{3}H]adenosine in the ratio of 12.5/1. [^{3}H]Adenosine-labeled material associated with protein was isolated by virtue of its resistance to ribonucleases T_2 and U_2. This material was released into the supernatant following incubation of the protein fraction with hydroxylamine, alkaline buffers, or 0.3 M KOH and was identified as oligo(ADP-ribose) with average chain length of 4–5 units by its hydrolysis to 5′-[^{3}H]AMP and phosphoribosyl[^{3}H]AMP with snake venom phosphodiesterase. 5′-[^{3}H]AMP was also released from the labeled protein fraction by alkali and snake venom phosphodiesterase suggesting ovum proteins are also mono(ADP-ribosylated). At the time of maximum [^{3}H]-adenosine incorporation, 24% of the label is present as oligo(ADP-ribose), 9% as the monomer, 47% as $(A)_{14}$ segments, and 20% is present at the 3′ terminus of RNA. [^{3}H]Adenosine, [^{3}H]ADP-ribose, and [^{3}H]cAMP were absent from the supernatant fraction of ova lysates but [^{3}H]NAD, the precursor of poly(ADP-ribose), [^{3}H]ATP, [^{3}H]ADP, and [^{3}H]AMP were present. The kinetics of [^{3}H]adenosine incorporation suggest that a relationship may exist between adenosine metabolism in the unfertilized ovum and its fertilizability.

Biochemical studies of fertilization and embryonic development have concentrated on the echinoderm and amphibian rather than mammals because of the difficulty in collecting large numbers of mammalian ova and embryos in a short time, and the lack of suitable media and methods for their in vitro culture. The technique for superovulation (Gates, 1971) has enabled the collection of larger numbers of ova and embryos, and the development of culture methods using chemically defined media which support embryonic development through the preimplantation stages (Brinster, 1972) has led to increasing numbers of studies on mammalian (usually mouse and rabbit) embryonic development (see Schultz & Church, 1975; Epstein, 1975). As yet, however, there have been few biochemical studies on the mammalian one-cell embryo or the unfertilized ovum.

At ovulation, the mouse ovum contains a store of maternal products synthesized during oogenesis. These include polysaccharides, a variety of enzymes, rRNA, tRNA (see Biggers & Stern, 1973; Engel & Franke, 1976) and, based on the presence of poly(A) (G. Stull, personal communication) and incorporation of guanosine into the 5′-terminal cap (Young, 1977), mRNA. After fertilization, a low level of RNA synthesis can be detected (Young et al., 1978), but the pronuclei

† From the Reproductive Biology Unit, Department of Obstetrics and Gynecology, Cornell University Medical College, New York, New York 10021. *Received January 4, 1978.* Supported by The Rockefeller Foundation and National Institutes of Health Grant HD 10230.

0006-2960/78/0417-1901$01.00/0

do not show RNA polymerase activity (Moore, 1975; Young et al., 1978), implying that maternal RNA is utilized for development of the zygote. The mouse ovum can be activated parthenogenetically in vitro or in vivo, and in favorable cases the embryo will survive beyond implantation (Kaufman, 1975). However unless the ovum is activated either by fertilization or parthenogenetically, ultrastructural changes suggestive of degeneration commence about 15 h after ovulation (Szollosi, 1971). The mechanism underlying the activation of the mouse ovum or its degeneration, and the role that the maternal products play in these processes as well as in the development of the zygote are unknown.

Ovulation occurs in the mouse, as in most mammals, after the oocyte has reached the metaphase stage of the second meiotic division. The chromosomes are in a condensed state and in agreement with the expectation that the DNA would be transcriptionally inactive neither guanosine nor uridine was incorporated into RNA (Young et al., 1978). However, adenosine was incorporated into cold trichloroacetic acid insoluble material (Young, 1976). This communication shows that adenosine is utilized by the unfertilized mouse ovum for polyadenylation of RNA and ADP-ribosylation[1] of proteins.

Materials and Methods

Collection and Labeling of Ova. Virgin random-bred Swiss female mice 7–12 weeks old were superovulated by intraperitoneal injection with pregnant mare's serum followed by human chorionic gonadotropin. Ova were collected and incubated in Whitten's medium in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 as previously described (Young et al., 1978). For kinetic experiments, ova were collected from up to 10 females at hourly intervals commencing 0–0.5 h post ovulation (the time of ovulation was taken as 13 h after the second injection) and divided into two groups. One group was incubated in medium containing [2,8-^{3}H]adenosine (30–50 Ci/mol) (New England Nuclear) at a concentration of 300 μCi/mL, and the second was placed in medium in which actinomycin D (Calbiochem) was present at 0.01 μg/mL. After incubation for 2 h at 37 °C each group of ova was washed in medium containing adenosine (0.1 mg/mL), transferred in batches of 18–40 to 0.1 M Tris-HCl (pH 7.5)–1% sodium dodecyl sulfate, and lysed by repeated freeze-thawing, and the cold trichloroacetic acid insoluble radioactivity in the lysate was measured as previously described (Young et al., 1978). Preparative labeling of large numbers of ova)up to 700) was carried out with gentle agitation on a reciprocating shaker in medium containing [^{3}H]adenosine at 500 μCi/mL.

Isolation of [^{3}H]Adenosine Labeled Material. After 2-h incubation with label, ova were washed 4–6 times with medium containing adenosine (0.1 mg/mL) and transferred to 0.2 mL of 0.1 M Tris-HCl (pH 7.2) containing 1% sodium dodecyl sulfate and 15 μg of yeast tRNA (Sigma). The ova were lysed by repeated freeze-thawing, and the labeled material was precipitated with 2 volumes of ethanol in the presence of 2% sodium acetate (pH 5) at −20 °C for 16 h. [^{3}H]Adenosine-labeled RNA was isolated from the labeled precipitate by resuspension of the precipitate in 0.1 M Tris-HCl (pH 8)–1% sodium dodecyl sulfate and then extraction with chloroform–phenol (Perry et al., 1972) or by the procedure of Brawerman (1973). The labeled RNA was recovered from the aqueous layer by ethanol precipitation in the presence of 2% sodium acetate (pH 5) and washed with 70% aqueous ethanol.

ADP-ribosylated proteins were isolated by incubation of the labeled precipitate with ribonucleases T_2 (10 units) (Sigma) and U_2 (1 unit) (Calbiochem) in 0.1 M sodium acetate (pH 5) and 5 mM EDTA for 12–16 h at 37 °C. The mixture was adjusted to 2% sodium acetate (pH 5) and [^{3}H]adenosine-labeled protein was precipitated with 2 volumes of ethanol at −20 °C for 16 h. Soluble labeled material was removed by four washes of the protein precipitate with cold 20% trichloroacetic acid followed by cold 70% aqueous ethanol. This procedure efficiently removed adenosine, 5′-AMP, and ADP from the protein pellet, but some [^{3}H]ATP was tenaciously retained.

Chemical and Enzyme Digestion. Poly(A) segments were released from the [^{3}H]adenosine-labeled RNA by incubation with ribonucleases A (Worthington) and T_1 (Calbiochem) as described (Rodriquez-Pousada & Hayes, 1976) except that sodium dodecyl sulfate was omitted and poly(A) (Miles; 3 A_{260} units) and yeast tRNA (10 μg) were added to the incubation mixture. Snake venom phosphodiesterase (Worthington) was used in 0.01 M $MgCl_2$, 0.1 M Tris-HCl, pH 7.5 or pH 8.8; 50 μg of the enzyme preparation did not convert 5′-AMP to adenosine after 3 h at 37 °C. Pronase (nuclease free, Calbiochem) was incubated at 10 mg/mL in 0.05 M Tris-HCl (pH 7.5) for 1 h before use; Pronase digestion was carried out in 0.1 M Tris-HCl (pH 7.5). Alkaline phosphatase from *E. coli* (Worthington) was used in 0.1 M Tris-HCl (pH 8). All enzyme incubations were performed at 37 °C.

Alkaline hydrolysis was carried out in 0.3 M KOH at 37 °C for 16–20 h. The hydrolysate was desalted with perchloric acid in the cold before examination by electrophoresis or chromatography. Reaction of the [^{3}H]adenosine-labeled protein with hydroxylamine was carried out with 0.5 M hydroxylamine in 0.1 M Tris-HCl (pH 7.5) at 37 °C for 1 h.

Electrophoresis and Chromatography. Paper electrophoresis was performed for 1.5–3 h at 18 V/cm in a Markem-Smith apparatus on Whatman No. 1 paper with either 0.05 M citrate (pH 5) or 0.05 M triethylammonium acetate–0.005 M EDTA (pH 5) as the buffer. Paper chromatography was carried out with Whatman No. 1 paper in solvent A. If paper chromatography were to be followed by paper electrophoresis, chromatography was stopped when the solvent front had moved 10 cm beyond the origin, the paper was dried, and electrophoresis carried out in the opposite direction (reverse electrophoresis) or in the same direction (forward electrophoresis) as chromatography. Thin-layer chromatography was carried out on either polyethylenimine impregnated cellulose or cellulose thin-layer sheets (Baker). Solvent systems were: (A) isopropyl alcohol–7.2 N ammonium hydroxide–water, 7:1:2 (v/v); (B) 1 M acetic acid; (C) 0.3 M LiCl–0.9 M acetic acid; (D) 0.75 M LiCl; (E) 1 M LiCl; (F) distilled water (pH 5); (G) step LiCl (Randerath & Randerath, 1964); (H) 0.15 M sodium borate–0.5 M boric acid; (I) 0.37 M sodium formate. Polyethylenimine cellulose thin-layer sheets were washed with 1 M LiCl and water before use. For two-dimensional polyethylenimine cellulose thin-layer chromatography, the sheet was developed in water, dried, and run in the same direction in solvent B until front had moved 3 cm and development continued in solvent C. A narrow strip containing marker adenosine, the fastest moving spot, was cut from the dried sheet which was then washed in anhydrous methanol and dried before chromatography in the second dimension in solvent G. Internal markers were always added. Radioactive areas were located in chromatograms with the aid of added markers and by measuring the radioactivity in 0.5-cm strips. Radioactivity was eluted from paper with 1 M ammonium hydroxide or 1 N

[1] Abbreviations used: ADP-ribose, adenosine diphosphate ribose; phosphoribosyl-AMP, 2′-(5″-phosphoribosyl)-5′-AMP; oligo(ADP-ribose), oligo(adenosine diphosphate ribose).

hydrochloric acid, and from polyethylenimine cellulose sheets with a solution of 0.05 M acetic acid, 0.3 M LiCl, 7 M urea before counting in a toluene based scintillation fluid (4 g of Omnifluor/L) (New England Nuclear) containing BBS3 (Beckman).

Results

[^3H]Adenosine Incorporation. Unfertilized mouse ova incorporated [^{3}H]adenosine into cold trichloroacetic acid insoluble material 1–6 h post ovulation (Figure 1). In the experiment shown, maximum incorporation occurred 2–4 h after ovulation, but in others this maximum was at 3–5 h after ovulation. The 1-h shift in the time of maximum incorporation is due to asynchrony of ovulation in the females, but in each of five experiments, the time of maximum [^{3}H]adenosine incorporation occurred either at 2–4 or 3–5 h after ovulation. Incorporation of [^{3}H]adenosine decreased shortly after the period of maximum incorporation falling to less than half the maximum value by 6 h after ovulation. This rapid decrease in incorporation was found in all experiments. Actinomycin D at a concentration (0.01 μg/mL) sufficient to inhibit the first cleavage of one-cell embryos by 50% had no effect on [^{3}H]-adenosine incorporation (Figure 1) but the antibiotic at a higher concentration (0.1 μg/mL) stimulated [^{3}H]adenosine incorporation.

The Nature of the [^3H]Adenosine-Labeled Material. Only 50–55% of the radioactivity in the crude lysates of ova incubated for 2 h with [^{3}H]adenosine 2, 3, or 4 h after ovulation became soluble in cold trichloroacetic acid after incubation with ribonuclease A, and about 34% of the radioactive material was resistant to 0.3 M KOH. Very little cold trichloroacetic acid insoluble [^{3}H]adenosine-labeled material was released into the aqueous layer when ova lysates were extracted with 80% aqueous phenol in the presence of 1% sodium dodecyl sulfate, but most (70%) of the labeled material in the aqueous layer was sensitive to ribonuclease; alkali resistant material was also present in the aqueous layer. Chloroform–phenol extraction (Perry et al., 1972) of [^{3}H]adenosine-labeled ova lysates released more labeled material into the aqueous phase than by extraction with phenol alone, but much radioactivity was still retained in the organic phase. This radioactive material could be recovered by dilution of the organic layer with water and removal of the organic solvent with ether. Incubation of the recovered labeled material with 0.3 M KOH released [^{3}H]AMP, but variable amounts of radioactivity remained at the origin on pH 5 electrophoresis of the hydrolysate showing that alkali resistant [^{3}H]adenosine-labeled material was present in the organic phase.

The [^{3}H]adenosine-labeled material could also be isolated from crude ova lysates by precipitation with 2 volumes of ethanol in the presence of 2% sodium acetate (pH 5). [^{3}H]ATP was tenaciously retained in the precipitate and repeated ethanol precipitation or washings with cold trichloroacetic acid failed to completely remove this contaminant. Characterization of the [^{3}H]adenosine-labeled material was carried out by digesting it with 0.3 M KOH or ribonuclease T_2 for 16 h at 37 °C. Paper electrophoresis at pH 5 of the digests showed that both alkali and ribonuclease T_2 hydrolyzed the labeled material to [^{3}H]AMP and [^{3}H]adenosine with the ratio of the former to the latter varying between 1.4 and 1.9/1 in different experiments; some radioactive material remained at the origin of the electrophorograms showing that labeled material was resistant to both alkali and the enzyme. These experiments suggest that [^{3}H]adenosine is incorporated into the 3′ terminus as well as into internal positions of RNA; in addition the label is incorporated into material which is not RNA and which appears to be associated with proteins.

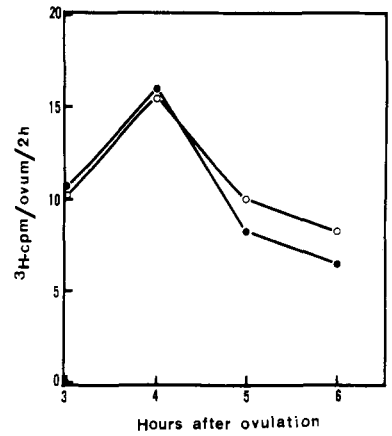

FIGURE 1: Time course of [^{3}H]adenosine incorporation by unfertilized mouse ova. The abscissa shows the time after ovulation that ova were removed from culture after a 2-h incubation as described in Materials and Methods. Each experimental point is the average value from 2 to 3 batches of 18–25 ova; the filter disc method was used for assay of [^{3}H]adenosine incorporation (Young et al., 1978). (●) No actinomycin D; (○) actinomycin D at 0.01 μg/mL.

The [^3H]Adenosine-Labeled RNA. Since DNA of the unfertilized ovum is in a condensed state, it is likely that [^{3}H]adenosine was incorporated into adjacent positions at the 3′ terminus of RNA. To determine if segments of contiguous AMP residues are indeed present, RNA was isolated by chloroform–phenol extraction of ova incubated with [^{3}H]-adenosine 2.5–4.5 h after ovulation and digested with a mixture of ribonucleases A and T_1. The results above suggested that the segments of [^{3}H]AMP residues would be small, average length of 2–4 residues, and the enzymic digest was therefore examined by paper chromatography in solvent A followed by reverse electrophoresis at pH 5. This procedure separated the digest into 4 radioactive peaks, two near the origin, one with the same mobility as ATP, and the fourth in the region of A_5 (Figure 2). The radioactive peak behind the origin was coincident with marker adenosine and after elution from the electrophorogram the radioactivity comigrated with adenosine when chromatographed on thin-layer sheets of cellulose in solvent F. This peak is therefore [^{3}H]adenosine.

The radioactive material with the same mobility as ATP also comigrated with ATP on polyethlyenimine cellulose thin-layer chromatography in solvent E. ATP was not a product of enzymic hydrolysis since it was detected when the [^{3}H]adenosine-labeled RNA preparation was examined by paper electrophoresis at pH 5. The nucleotide appeared to be tenaciously retained by the RNA as repeated washings with cold 10% trichloroacetic acid failed to completely remove it from the RNA.

The radioactive material in the peak near the origin was recovered by elution with 1 M ammonium hydroxide and hydrolyzed with 0.3 M KOH. Chromatography of the hydrolysate on thin-layer sheets of polyethylenimine cellulose in solvent H showed that this material was completely hydrolyzed to 3′-[^{3}H]AMP and [^{3}H]adenosine with the ratio of the former to the latter of 12.5/1. [^{3}H]Adenosine and 3′-[^{3}H]AMP were also obtained when the material in the peak near A_5 was hydrolyzed with 0.3 M KOH.

These experiments show that oligo([^{3}H]A) segments with [^{3}H]adenosine at the 3′ terminus are liberated by ribonucleases A and T_1 from [^{3}H]adenosine-labeled RNA and suggest that [^{3}H]adenosine is incorporated into the RNA of unfertilized mouse ova by sequential addition to the 3′ terminus.

The [^3H]Adenosine-Labeled Protein Component. The re-

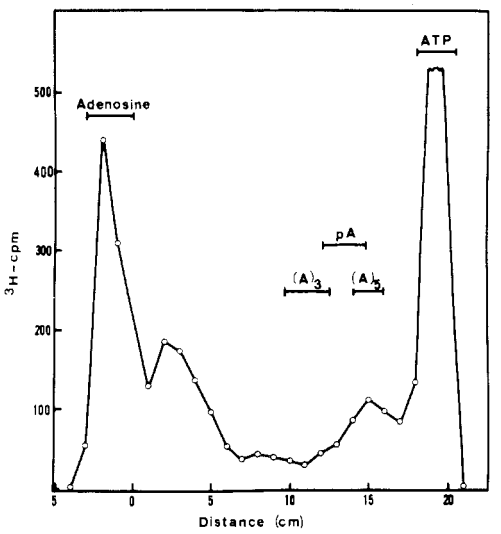

FIGURE 2: Electrophorogram of ribonuclease A and T_1 digest of RNA isolated from unfertilized ova incubated 2.5–4.5 h after ovulation with [^{3}H]adenosine. [^{3}H]Adenosine-labeled RNA was isolated from ova lysates and digested with ribonucleases A and T_1 as described in Materials and Methods. The enzymic digest was chromatographed on Whatman No. 1 paper in solvent A. When the front had moved 10 cm beyond the origin, the chromatogram was dried and electrophoresis carried out in 0.05 M triethylammonium acetate–0.005 M EDTA, pH 5, in the reverse direction. The dried electrophorogram was cut into 1-cm strips and placed in scintillation fluid (4 g of Omnifluor/L of toluene) and the radioactivity measured. [^{3}H]Adenosine-labeled material was eluted from the paper strips with 1 M NH_4OH. The origin is 0; chromatography direction is to the left.

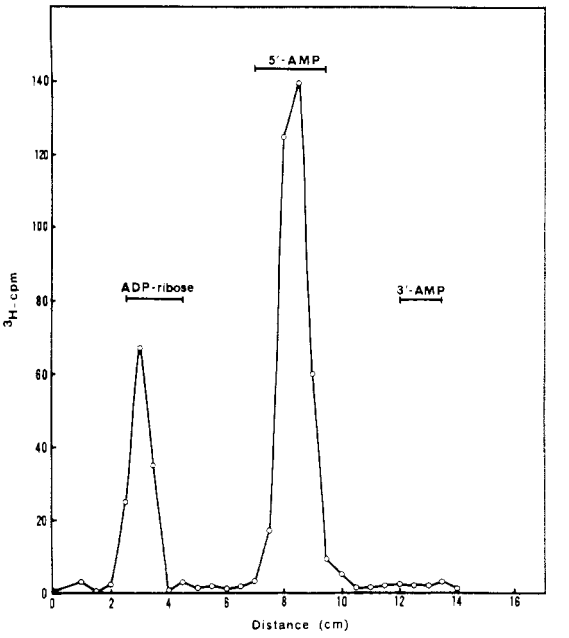

FIGURE 3: Chromatogram of snake venom phosphodiesterase digest of [^{3}H]adenosine-labeled ovum protein. [^{3}H]Adenosine-labeled protein isolated from unfertilized ova incubated with [^{3}H]adenosine 2.5–4.5 h after ovulation was isolated and digested with Pronase as described in the text. The phenol-extracted Pronase digest was incubated with snake venom phosphodiesterase (50 μg), ATP (30 μg) in 0.1 M Tris-HCl, pH 8.9, and 0.01 M $MgCl_2$ for 3 h at 37 °C and the digest chromatographed on polyethylenimine cellulose thin-layer sheets in solvent H.

tention of [^{3}H]adenosine-labeled material in the organic phase after chloroform–phenol extraction of unfertilized ova lysates, and the presence of alkali resistant labeled material in these lysates suggest that [^{3}H]adenosine was incorporated into a non-RNA component which is associated with protein. In order to identify this component, lysates of ova labeled with [^{3}H]-adenosine 2.5–4.5 h after ovulation were adjusted to 2% sodium acetate (pH 5) and the [^{3}H]adenosine-labeled material precipitated with 2 volumes of ethanol at −20 °C. The precipitate was digested with a mixture of ribonucleases T_2 and U_2 to remove labeled RNA; the labeled protein precipitated with ethanol at −20 °C and then washed with cold 20% trichloroacetic acid. As found in the isolation of labeled RNA washing the protein preparation with trichloroacetic acid removed all the soluble contaminants except [^{3}H]ATP. This labeled protein preparation containing some [^{3}H]ATP was digested with Pronase, the enzyme removed by chloroform–phenol extraction, and the [^{3}H]adenosine-labeled material in the aqueous layer was incubated with snake venom phosphodiesterase. Thin-layer chromatography of this digest on polyethylenimine cellulose in solvent H resolved the radioactive material in the digest into two radioactive peaks (Figure 3). The same result was obtained if the chromatogram was developed in solvent I or solvent B until the front had moved 9–10 cm followed by solvent C. Paper electrophoresis at pH 5 also resolved the digest into two radioactive peaks. The faster moving peak in the thin-layer chromatograms had the same R_f as marker 5′-AMP and the slower moving peak the same R_f as marker phosphoribosyl-AMP, which was obtained by hydrolysis of poly(ADP-ribose) with snake venom phosphodiesterase. With paper electrophoresis the faster peak had the same mobility as marker phosphoribosyl-AMP and the slower peak the same mobility as 5′-AMP. The mobility of phosphoribosyl-AMP with respect to ADP-ribose and 5′-AMP on paper electrophoresis and thin-layer chromatography is shown in Table I.

TABLE I: Mobility of Phosphoribosyl-AMP Relative to 5′-AMP and ADP-ribose on Polyethylenimine Cellulose Thin-Layer Chromatography and Paper Electrophoresis.

Thin-layer chromatography	Electrophoresis	Relative mobility 5′-AMP	Relative mobility ADP-ribose
Solvent B, then C		0.36	1.13
Solvent H		0.39	0.89
Solvent I		0.29	0.90
	pH 5	1.65	1.11

Identification of phosphoribosyl-[^{3}H]AMP in the snake venom phosphodiesterase digest shows that [^{3}H]adenosine is incorporated into the polymer, poly([^{3}H]ADP-ribose), by unfertilized ova.

In principle, the length of the polymer can be determined from the ratio of phosphoribosyl-AMP to 5′-AMP in the snake venom phosphodiesterase digest, but, because ATP is also hydrolyzed to AMP by this enzyme and [^{3}H]ATP is present in the protein preparation, the present experiments cannot give an indication of the polymer size. Nevertheless in these experiments, the amount of 5′-[^{3}H]AMP found in each digest was far in excess of that expected from hydrolysis of the contaminating [^{3}H]ATP and poly([^{3}H]ADP-ribose), even assuming that the dimer ([^{3}H]ADP-ribose)$_2$ was attached to ovum proteins. Possible sources of the excess 5′-[^{3}H]AMP in the digest are [^{3}H]adenosine-labeled RNA, which was not completely degraded by ribonucleases T_2 and U_2, and [^{3}H]-ADP-ribose, since this nucleotide is cleaved to 5′-[^{3}H]AMP by snake venom phosphodiesterase even when covalently linked to proteins (Honjo et al., 1971).

To determine if undigested [^{3}H]adenosine-labeled RNA is a source of the excess [^{3}H]AMP, lysates of ova labeled 2.5–4.5 h with [^{3}H]adenosine were digested with ribonucleases T_2 and U_2; the unhydrolyzed labeled material was washed with

cold 20% trichloroacetic acid, incubated for 1 h with Pronase, and then incubated with 0.3 M KOH for 16 h at 37 °C. Paper electrophoresis at pH 5 resolved the alkaline hydrolysate into [^{3}H]AMP and poly([^{3}H]ADP-ribose) but polyethylenimine cellulose thin-layer chromatography in solvent H showed that the [^{3}H]AMP was the 5′ isomer and 3′-[^{3}H]AMP was absent from the alkaline hydrolysate. Therefore [^{3}H]adenosine-labeled RNA was completely removed by ribonucleases T_2 and U_2 and it is not the source of the excess 5′-[^{3}H]AMP released from the ovum proteins by snake venom phosphodiesterase. On the other hand, ADP-ribose but not ATP was hydrolyzed to 5′-AMP when incubated for 16 h with 0.3 M KOH at 37 °C (Kaplan et al., 1951); hydrolysis was essentially complete by 2 h. Thus it is most likely that ADP-ribose covalently linked to protein is the source of the excess 5′-AMP.

Figure 3 shows that the [^{3}H]adenosine-labeled material associated with ovum protein is hydrolyzed completely to phosphoribosyl-[^{3}H]AMP and 5′-[^{3}H]AMP by snake venom phosphodiesterase. When the ovum protein was digested with snake venom phosphodiesterase without prior degradation with Pronase, not all the radioactivity was released into the supernatant, and ovum protein recovered from the digest by precipitation with ethanol and 2% sodium acetate (pH 5) followed by washing with cold trichloroacetic acid was still radioactive; paper electrophoresis at pH 5 showed that [^{3}H]ATP was absent. After incubation of the recovered protein with Pronase and digestion of the phenol-extracted Pronase digest with snake venom phosphodiesterase, the [^{3}H]adenosine-labeled material which was released was identified by thin-layer chromatography in solvent H as 5′-[^{3}H]AMP. Therefore the [^{3}H]adenosine-labeled material associated with mouse ovum proteins can be completely cleaved by snake venom phosphodiesterase to either phosphoribosyl-[^{3}H]AMP and 5′-[^{3}H]AMP, or to 5′-[^{3}H]AMP alone. This suggests that [^{3}H]adenosine is incorporated into mouse ovum proteins as mono(ADP-ribose) and poly(ADP-ribose).

Since the precursor of poly(ADP-ribose) is NAD, the composition of the soluble fraction was studied to determine if [^{3}H]adenosine is converted to [^{3}H]NAD. The soluble fraction from unfertilized ova incubated with [^{3}H]adenosine 2.5–4.5 h post ovulation was examined by paper chromatography in solvent A followed either by forward or reverse paper electrophoresis at pH 5, by thin-layer chromatography on polyethylenimine cellulose in solvent D or solvent B until the front had reached 2–3 cm and development continued in solvent C, and by two-dimensional thin-layer chromatography. These procedures showed that the nucleotides ATP, ADP, AMP, and NAD but not ADP-ribose or cAMP were present in ova lysates. The percentage of [^{3}H]NAD in the lysate determined in three experiments ranged between 0.34% and 0.54%, and, although the percentage of ATP and ADP in these experiments varied between 12% and 48%, the ratio ATP/ADP remained constant (1.53–1.67). [^{3}H]Adenosine was not found in ova lysates but some (1.7–2.5%) unknown radioactive material with a mobility similar to adenosine was found in the chromatograms and electrophorograms. Two-dimensional thin-layer chromatography or paper chromatography followed by electrophoresis in the reverse direction also partially separated some radioactive material from [^{3}H]AMP. The identity of this material is also unknown.

Polymer Length and Release from Protein. The absence of the polymer in the aqueous layer after chloroform–phenol extraction of ova lysates and its release by Pronase digestion show that the polymer is associated with, and perhaps attached by, covalent bonds to ovum proteins. Since poly(ADP-ribose) is widely found covalently attached to proteins by bonds which

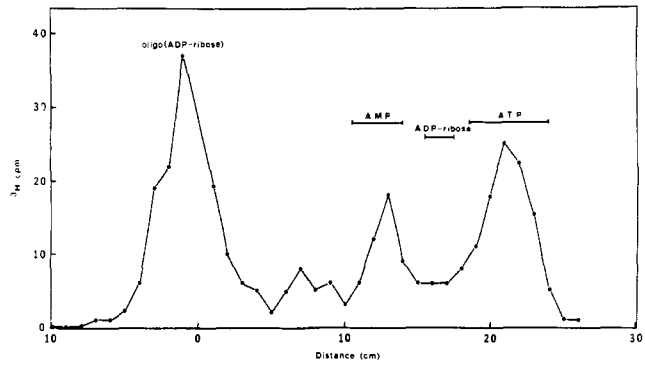

FIGURE 4: Electrophorogram of [^{3}H]adenosine-labeled material released from labeled ovum protein by hydroxylamine. [^{3}H]Adenosine-labeled RNA was removed from lysates of unfertilized ova which had been incubated with [^{3}H]adenosine from 2.5 to 4.5 h after ovulation by digestion with ribonucleases T_2 and U_2 as described in Materials and Methods. After washing with cold trichloroacetic acid and ethanol, the labeled protein was incubated with 0.5 M hydroxylamine in 0.1 M Tris-HCl, pH 7.5, for 1 h at 37 °C. The protein was removed by precipitation with ethanol at pH 5 and the supernatant examined by paper chromatography followed by electrophoresis as described in Figure 2. The origin is 0 and the direction of chromatography is to the left. Radioactive material was recovered by elution with 1 M NH_4OH.

vary in their sensitivity toward hydroxylamine and alkaline conditions (see Hilz & Stone, 1976; Hayaishi & Ueda, 1977), the stability of the [^{3}H]adenosine-labeled ovum proteins toward these reagents was investigated. [^{3}H]Adenosine-labeled RNA was removed from lysates of ova labeled with [^{3}H]-adenosine 2.5–4.5 h after ovulation by digestion with ribonucleases T_2 and U_2. The cold trichloroacetic acid washed labeled protein was incubated with 0.5 M hydroxylamine (pH 7.5), and after adjustment of the pH to 5 with 20% sodium acetate (pH 5) the protein was precipitated with 2 volumes of ethanol at −20 °C. Examination of the supernatant by paper chromatography in solvent A followed by electrophoresis at pH 5 in the reverse direction showed that three radioactive components were present (Figure 4). The component with the highest mobility was [^{3}H]ATP which was present as a contaminant in the labeled protein. A second component was identified as [^{3}H]AMP since it comigrated with marker AMP and was converted to [^{3}H]adenosine on incubation with alkaline phosphatase. The remaining radioactive component, at the origin of the electrophorogram, was hydrolyzed by snake venom phosphodiesterase to give phosphoribosyl-[^{3}H]AMP (237 cpm) and 5′-[^{3}H]AMP (74 cpm), a ratio of 3.2/1. Therefore the radioactive material at the origin is an oligomer of ADP-ribose of average chain length 4–5 units. A similar result was obtained when the [^{3}H]adenosine-labeled protein preparation was incubated at 37 °C with 0.1 M Tris-HCl (pH 8.5) or 0.1 M $(NH_4)_2CO_3$ (pH 9.2). Although the oligomer was released from protein after incubation for 1 h, protein recovered after 4 h still contained attached oligo(ADP-ribose). Similarly, protein recovered after 1-h incubation with hydroxylamine continued to release the oligomer when incubated further with $(NH_4)_2CO_3$ (pH 9.2). The association between oligo(ADP-ribose) and ovum protein is therefore sensitive to hydroxylamine and alkaline conditions in agreement with the observations of other workers (Nishizuka et al., 1968, 1969; Adamietz & Hilz, 1976).

The covalent bond joining mono(ADP-ribose) to proteins is labile to either acid, alkali, or hydroxylamine (Stone & Hilz, 1975; Goff, 1974; Smith & Stocken, 1973; Skórko et al., 1977) or is stable to all of these reagents (Honjo et al., 1971). Figure 4 shows that neither [^{3}H]ADP-ribose nor its oxime was released from [^{3}H]adenosine-labeled proteins when the protein

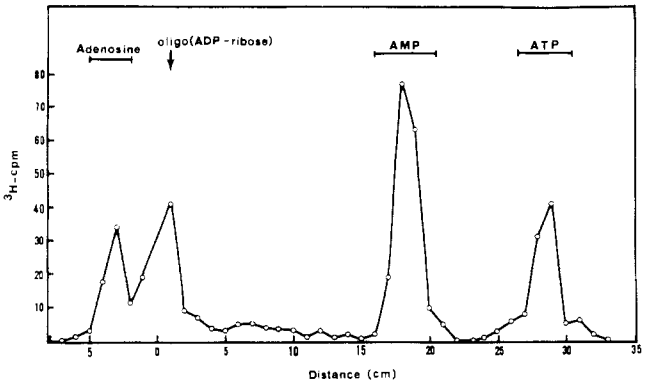

FIGURE 5: Electrophorogram of alkaline hydrolysate of [^{3}H]adenosine-labeled unfertilized mouse ova. Mouse ova after incubation with [^{3}H]adenosine 2.4–4.5 h after ovulation were tranferred immediately to cold 20% trichloroacetic acid and washed four times with cold trichloroacetic acid followed by cold ethanol. The labeled ova were incubated with 0.3 M KOH for 16 h at 37 °C, and after desalting with $HClO_4$ the hydrolysate was examined by paper chromatography in solvent A followed by electrophoresis as described in Figure 2. Direction of chromatography is to the left and 0 is the origin.

preparation was incubated with 0.5 M hydroxylamine. [^{3}H]ADP-ribose was also absent from the supernatant when labeled ovum proteins were incubated for up to 6 h with Tris-HCl (pH 8.5) or 0.1 M $(NH_4)_2CO_3$, although 5′-[^{3}H]AMP was present. It is unlikely that [^{3}H]ADP-ribose was liberated from the labeled protein and then rapidly hydrolyzed to 5′-[^{3}H]AMP since very little hydrolysis of ADP-ribose was found after 5 h at 37 °C in 0.1 M Tris-HCl (pH 8.5). Similarly, [^{3}H]ADP-ribose was not detected when [^{3}H]adenosine-labeled ovum proteins were treated with HCl under conditions (0.1 N HCl 16 h at 37 °C; 1 N HCl, 7 min at 90 °C; 1 N HCl, 4 h at 37 °C) which, in control experiments, did not completely hydrolyze ADP-ribose to 5′-AMP. Therefore the bond joining mono(ADP-ribose) to ovum proteins is stable to hydroxylamine and acid and alkaline conditions, a result similar to that reported by Honjo et al. (1971). However, still unexplained is the release of 5′-[^{3}H]AMP into the supernatant when labeled ovum proteins are subjected to slightly alkaline conditions.

To investigate the possibility that degradation of poly(ADP-ribose) occurred during isolation of labeled proteins, ova were added to and washed with cold 10% trichloroacetic acid immediately after labeling with [^{3}H]adenosine. The ova were then washed with ethanol, dried, and hydrolyzed with 0.3 M KOH at 37 °C. Paper chromatography in solvent A followed by electrophoresis at pH 5 in the reverse direction separated the hydrolysate into four radioactive peaks (Figure 5). The radioactive material in the peak behind the origin was identified as adenosine as it comigrated with adenosine marker in this system as well as on cellulose thin-layer sheets in solvent F. The material in the peak at the origin was shown to be oligo(ADP-ribose) by its hydrolysis to phosphoribosyl-[^{3}H]AMP and 5′-[^{3}H]AMP by snake venom phosphodiesterase, while the material in the peak with the same mobility as [^{3}H]AMP was identified as a mixture of 3′-[^{3}H]AMP and 5′-[^{3}H]AMP by thin-layer chromatography on polyethylenimine cellulose in solvent H. Snake venom phosphodiesterase hydrolyzed the material in the peak which comigrated with ATP to 5′-[^{3}H]-AMP thereby showing this material was [^{3}H]ATP. A similar result was obtained when the alkaline hydrolysate was analysed by thin-layer chromatography on polyethylenimine cellulose in solvent H. The size of the oligomer found in these experiments was the same as that found in the previous experiments showing that the oligomer size in the ovum protein is 4–5 units

TABLE II: Percentage Distribution of [^{3}H]Adenosine in Alkaline Hydrolysate of [^{3}H]Adenosine-Labeled Mouse Ova.

Compound	Percentage[a]
Adenosine	20.8
3′-AMP	46.7
5′-AMP	8.9
Oligo(ADP-ribose)	23.6

[a] Average of two experiments.

and that degradation did not occur during its isolation. Further, the presence of 5′-[^{3}H]AMP in the hydrolysate suggests that the monomer is also present in ovum proteins.

The percentages of [^{3}H]adenosine, [^{3}H]AMP, and phosphoribosyl-[^{3}H]AMP present in the hydrolysate are shown in Table II. Since [^{3}H]adenosine is derived from 3′-terminal adenosine, 67.5% of the nucleoside is incorporated into RNA, while 32.5% is incorporated into protein, 23.6% as oligo(ADP-ribose) and 8.9% as mono(ADP-ribose).

Discussion

Adenylation of RNA occurs in the newly fertilized ova of rabbit (Schultz, 1975) and mouse (Young, unpublished experiments), and the present experiments show that it also occurs in the unfertilized mouse ovum. Whether the adenylation of ovum RNA represents a lengthening or turnover of their poly(A) segments is unknown. The biological role of cytoplasmic adenylation of RNA, first observed in mammalian cells, is still unresolved (see Brawerman, 1976), but, since the chromosomes of the unfertilized ovum are inactive, the ovum may be a suitable system for the study of this biochemical event. Modification of proteins by ADP-ribosylation is also widespread (see Hilz & Stone, 1976; Hayaishi & Ueda, 1977), although the phenomenon has not previously been observed in mammalian ova or embryos. The covalent attachment of mono(ADP-ribose) or poly(ADP-ribose) to nuclear proteins, eukaryotic elongation factor 2, and *E. coli* RNA polymerase after T_4 phage infection results in a functional change in the modified proteins and this has led to the belief that ADP-ribosylation of proteins is involved in the regulation of cell growth, chromatin function, and protein and nucleic acid synthesis (Hilz & Stone, 1976; Hayaishi & Ueda, 1977).

Of interest in this regard is the time course of [^{3}H]adenosine incorporation by unfertilized ova. While [^{3}H]adenosine incorporation is high soon after ovulation, the period of maximum incorporation occurs 3–5 h after ovulation (Figure 1) when 47% of the [^{3}H]adenosine is incorporated into poly(A) segments and 33% is utilized for ADP-ribosylation of proteins (Table II). The level of [^{3}H]adenosine incorporation then declines commencing about 6 h after ovulation. The relative distribution of the label into each of the macromolecular species at this period after ovulation is not known at present, but, because the radioactivity incorporated by ova is only partially sensitive to alkali or ribonuclease A, it is likely that adenylation of RNA and ADP-ribosylation of proteins are occurring at a reduced level. Actinomycin D has no effect on cytoplasmic adenylation of RNA (see Brawerman, 1976) but it does inhibit, by virtue of its ability to bind to DNA, the partially purified rat liver chromatin poly(ADP-ribose) polymerase which has an absolute requirement for DNA for polymerase activity (Yoshihara, 1972). However, the antibiotic enhances the activity of the polymerase in intact nuclei (Claycomb, 1976), and the stimulation of [^{3}H]adenosine incorporation observed (Figure 1) suggests that the enzyme is

active in the ovum for at least 6 h after ovulation. Since the ovulated ovum does not divide, synthesize DNA or RNA, or undergo chromatin condensation, ADP-ribosylation of unfertilized ovum protein(s) may be associated with modification or inhibition of enzyme activity, and thus specific metabolic changes may be occurring in the unfertilized ovum at the time of maximum [^{3}H]adenosine incorporation and also 6 h post ovulation.

The kinetics of [^{3}H]adenosine incorporation by the mouse ovum parallels the time of its fertilization and the period during which it is able to support embryonic development. Mouse ova are not normally fertilized until 3–5 h after ovulation (Braden & Austin, 1954; Braden, 1959), which is the period of maximum [^{3}H]adenosine incorporation, and are capable of being fertilized normally for a further 7–9 h, i.e., as late as 10–12 h after ovulation (Marston & Chang, 1964; Iwamatsu & Chang, 1971). A decrease in ovum fertilizability occurs, however, as early as 7–8 h after ovulation (Iwamatsu & Chang, 1971; Wolf & Hamada, 1976) at the time when the level of [^{3}H]adenosine incorporation is low. Although some ova can be fertilized as late as 18–20 h after ovulation, a male pronucleus either will not form in these ova and/or only a few develop (Marston & Chang, 1964; Iwamatsu & Chang, 1971). In other mammals similarly, fertilization toward the end of estrus is correlated with chromosomal abnormalities, abnormal development of fetuses, and spontaneous abortion (Runner & Palm, 1953; Hammond, 1934; Chang, 1952; Blandau & Young, 1939; Blandau & Jordon, 1941; Austin, 1970; Lanman, 1968; Guerrero & Rojas, 1975). Degenerative changes at the ultrastructural level are not observed in the mouse ova until about 2–4 h after they cease to be fertilizable (Szollosi, 1971). Thus biochemical changes associated with ovum degeneration precede ultrastructural alterations and may occur as early as 7–8 h after ovulation when a decrease in fertilizability is noted and when the level of [^{3}H]adenosine incorporation falls. The nature of the cytoplasmic elements in the ovum which control fertilizability and the ability to support embryonic development are unknown, but the kinetics of [^{3}H]adenosine incorporation point to a possible relationship between this and ADP-ribosylation of proteins and/or adenylation of RNA.

The delay of 3–5 h before mouse ova are fertilized may result from the kinetics of sperm transport to the site of fertilization (Braden & Austin, 1954; Zamboni, 1970a). On the other hand this may be related to post-ovulation maturational changes in the ovum since modifications of the structure and arrangement of the chromosomes, and an increase in the number of cortical granules have been observed in the period between ovulation and sperm penetration (Zamboni, 1970b). The correspondence between the time of fertilization and the period of maximum [^{3}H]adenosine incorporation suggests that a relationship may exist between these two events. Whether the ultrastructural changes in the ovulated ovum or the high level of [^{3}H]adenosine incorporation have any relevance for fertilizability or postfertilization development remains to be determined.

Acknowledgment

The gift of poly(ADP-ribose) from L. Burzio is gratefully acknowledged.

References

Adamietz, P., & Hilz, H. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357,* 527.

Austin, C. R. (1970) *J. Reprod. Fertil., Suppl. 12,* 39.

Biggers, J. D., & Stern, S. (1973) in *Advances in Reproductive Physiology* (Bishop, M. W. H., Ed.) p 1, The Gresham Press, Old Working, Surrey, England.

Blandau, R. J., & Jordon, E. S. (1941) *Am. J. Anat. 68,* 275.

Blandau, R. J., & Young, W. C. (1939) *Am. J. Anat. 64,* 303.

Braden, A. W. H. (1959) *Symp. Genet. Biol. Ital. 9,* 1.

Braden, A. W. H., & Austin, C. R. (1954) *Aust. J. Biol. Sci. 7,* 522.

Brawerman, G. (1973) *Methods Cell Biol. 7,* 1.

Brawerman, G. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 17,* 118.

Brinster, R. L. (1972) in *Growth, Nutrition and Metabolism of Cells in Culture* (Rothblat, G. H., & Cristofalo, V. J. Eds.) Vol. 2, p 251, Academic Press, New York, N.Y.

Chang, M. C. (1952) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 11,* 24.

Claycomb, W. C. (1976) *Biochem. J. 154,* 387.

Engel, W., & Franke, W. (1976) *Curr. Top. Pathol. 62,* 29.

Epstein, C. J. (1975) *Biol. Reprod. 12,* 82.

Gates, A. H. (1971) in *Methods in Mammalian Embryology* (Daniel, J. C., Jr., Ed.) p 64, W. H. Freeman, San Francisco, Calif.

Goff, C. G. (1974) *J. Biol. Chem. 249,* 6181.

Guerrero, R., & Rojas, O. L. (1975) *N. Engl. J. Med. 293,* 573.

Hammond, J. (1934) *J. Exp. Biol. 11,* 140.

Hayaishi, O., & Ueda, K. (1977) *Annu. Rev. Biochem. 46,* 95.

Hilz, H., & Stone, P. (1976) *Rev. Physiol. Biochem. Pharmacol. 76,* 1.

Honjo, J., Nishizuka, Y., Kato, I., & Hayaishi, O. (1971) *J. Biol. Chem. 246,* 4251.

Iwamatsu, T., & Chang, M. C. (1971) *J. Reprod. Fertil. 26,* 197.

Kaplan, N. O., Colowick, S. P., & Banes, C. C. (1951) *J. Biol. Chem. 191,* 461.

Kaufman, M. H. (1975) in *The Early Development of Mammals* (Balls, M., & Wild, A. E., Eds.) p 25, Cambridge University Press, Cambridge, England.

Lanman, J. T. (1968) *N. Engl. J. Med. 19,* 1047.

Marston, J. H., & Chang, M. C. (1964) *J. Exp. Zool. 155,* 137.

Moore, G. P. M. (1975) *J. Embryol. Exp. Morphol. 34,* 291.

Nishizuka, Y., Ueda, K., Honjo, T., & Hayaishi, O. (1968) *J. Biol. Chem. 243,* 3765.

Nishizuka, Y., Ueda, K., Yoshikara, Yamamura, H., Takeda, M., & Hayaishi, O. (1969) *Cold Spring Harbor Symp. Quant. Biol. 34,* 781.

Perry, R. P., LaTorre, J., Kelley, D. E., & Greensberg, J. R. (1972) *Biochim. Biophys. Acta 262,* 220.

Randerath, E., & Randerath, K. (1964) *J. Chromatogr. 16,* 126.

Rodriguez-Pousada, C., & Hays, D. H. (1976) *Eur. J. Biochem. 71,* 117.

Runner, M. N., & Palm, J. (1953) *J. Exp. Zool. 124,* 203.

Schultz, G. A. (1975) *Dev. Biol. 44,* 270.

Schultz, G. A., & Church, R. B. (1975) in *The Biochemistry of Animal Development* (Weber, R. Ed.) Vol. 3, p 47, Academic Press, New York, N.Y.

Skórko, R., Zillig, W., Rohrer, H., Fujiki, H., & Mailhammer, R. (1977) *Eur. J. Biochem. 79,* 55.

Smith, J. A., & Stocken, C. A. (1973) *Biochem. Biophys. Res. Commun. 54,* 297.

Stone, P. R., & Hilz, H. (1975) *FEBS Lett. 57,* 209.

Szollosi, D. (1971) *Am. J. Anat. 130,* 209.

Wolf, D. P., & Hamada, M. (1976) *J. Reprod. Fertil. 48,*

213.
Yoshihara, K. (1972) *Biochem. Biophys. Res. Commun. 47,* 119.
Young, R. J. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1690.
Young, R. J. (1977) *Biochem. Biophys. Res. Commun. 76,* 32.
Young, R. J., Sweeney, K., & Bedford, J. M. (1978) *J. Embryol. Exp. Morphol.* (in press).
Zamboni, L. (1970a) *Morphology of Mammalian Fertilization,* p 136, Harper & Row, New York, N.Y.
Zamboni, L. (1970b) *Biol. Reprod. 2,* 44.

Kinetic Analysis of Cooperativity in Tubulin Polymerization in the Presence of Guanosine Di- or Triphosphate Nucleotides†

Marie-France Carlier and Dominique Pantaloni*

ABSTRACT: In vitro polymerization of pig brain tubulin, highly purified and deprived of microtubule-associated proteins, was followed by turbidimetry. Treatment of the data yielded the relation existing between the observed turbidity and the amount of polymer formed. This allowed a kinetic analysis, according to Oosawa's theories, of the polymerization process, which consisted of a slow spontaneous nucleation followed by the growth process. The apparent elongation rate constant was closely related to the nucleation process and exhibited a highly cooperative variation with tubulin concentration. The cooperativity was indicative of the size of the nucleus which appears to remain the same whether sheets or microtubules are formed. Magnesium ions appear to play a role in the polymorphism of tubulin polymers, the proportion of microtubules to sheets increasing with magnesium ion concentration. From kinetic experiments evidence was provided for GDP binding in competition with GTP, with a sixfold lower affinity. The tubulin–GDP complex could participate in microtubules elongation, but was not able to form nuclei. The critical concentration of tubulin in the presence of GDP was roughly twice as high as in the presence of GTP.

Highly pure tubulin from mammalian brain is able to polymerize in vitro in the absence of any associated protein, and in a variety of buffers containing magnesium ions, GTP and glycerol (Lee & Timasheff, 1975, 1977). A thorough thermodynamic study of the equilibrium microtubules ⇌ tubulin has been performed under these medium conditions, but until now the kinetics of polymerization has not been used to obtain information about the mechanism and pathways of pure tubulin self-assembly. Turbidimetry measurements of pure tubulin self-assembly have been analyzed in this work with the kinetic theories of protein polymerization developed by Oosawa.

From the data found in the literature, GTP seems to play a puzzling role in tubulin assembly: GTP is necessary for assembly but is hydrolyzed during polymerization and at the ends of microtubules (Maccioni & Seeds, 1977; David-Pfeuty et al., 1977). Isolated microtubules contain only GDP at the exchangeable (E) site (Berry & Shelanski, 1972; Jacobs et al., 1974) and seem to be stable. Two classes of models can account for this result: either GDP is able to promote microtubules assembly, or GTP hydrolysis is necessary for polymerization. However, Weisenberg's experiments (Weisenberg et al., 1976) do not agree with the former hypothesis, while data from other authors indicate that nonhydrolyzable GTP analogues can induce polymerization, which eliminates the latter hypothesis (Kobayashi, 1974; Kobayashi & Simizu, 1976; Arai & Kaziro, 1976). In this paper, attempts have been made to clarify some of these points by a kinetic analysis of the observed effects of GTP and GDP on tubulin polymerization.

Materials and Methods

Reagents. 2-*N*-Morpholinoethanesulfonic acid (Mes)[1] was purchased from Calbiochem, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) from Sigma, and fibrous cellulose phosphate P 11 from Whatman. Guanosine 5′-triphosphate trilithium salt from Boehringer and guanosine 5′-diphosphate sodium salt I from Sigma were used after checking purity by chromatography on PEI-cellulose Polygram cell 300 (Macherey & Nagel). All other reagents were Merck's analytical grade. Radioactive nucleotides were purchased from Amersham.

Tubulin Purification. Tubulin was purified from fresh pig brain according to Shelanski et al. (1973) by three consecutive cycles of polymerization. Purification was achieved by passage of this tubulin preparation through a phosphocellulose column equilibrated in 0.05 M *N*-morpholinoethanesulfonic acid (Mes) buffer, pH 6.6, containing 0.25 mM $MgCl_2$, 0.5 mM EGTA, and 0.1 mM GTP. Tubulin was thus separated from the microtubule associated proteins (Weingarten et al., 1975). Purity of tubulin was checked by polyacrylamide gradient gel electrophoresis on PAA 4-30 Pharmacia Slabs in $NaDodSO_4$ buffer, pH 8.2. To the eluted tubulin fraction was then added 3.4 M glycerol and this solution was concentrated using an Amicon concentrating cell Model 52 equipped with a PM 30 Diaflo membrane. The resulting tubulin solution (5–10

† From the Laboratoire d'Enzymologie, CNRS, 91190 Gif-sur-Yvette, France. *Received October 5, 1977.* Supported by Centre National de la Recherche Scientifique, RCP No. 386, ATP 2303, and Délégation Générale à la Recherche Scientifique et Technique, ACC No. 76 711 76.

[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/78/0417-1908$01.00/0