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AN ATP-INCORPORATING ENZYME FROM RAT BRAIN

I SOLUBILIZATION AND REQUIREMENTS

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SUMMARY

An ATP-incorporating enzyme has been solubilized from a nonnuclear particulate fraction of rat brain with the nonionic detergent Triton X-100 The enzyme activity is not affected by deoxyribonuclease or ribonuclease, and is markedly stimulated by GTP and, to a lesser extent, by the other nucleoside triphosphates There is an absolute requirement for a heat-stable cofactor present in the same enzyme extract This cofactor has been separated from the enzyme by means of ethanol fractionation and appears to be a compound of relatively low molecular weight since it is dialyzable and heat-stable Its activating effect is lost after incubation with alkaline phosphatase The product of the reaction is not hydrolyzed by ribonuclease or deoxyribonuclease but is partially degraded by phosphodiesterase A rapid nonenzymic degradation of the product occurs in neutral or moderately alkaline conditions A significant fraction of the fragments released by alkaline hydrolysis behaves as though they were more highly charged than ADP

INTRODUCTION

The incorporation of ^{14}C-labeled ATP into acid-insoluble material catalyzed by mammalian tissues is generally attributed to enzymes which synthesize polynucleotides, such as RNA polymerases[1-4] and poly-A synthetases[5,6] More recently the formation of a polymer of adenosine diphosphoribose has been described in liver[7-9]

In this paper we report the presence of an enzyme system from rat brain which catalyzes the incorporation of radioactive ATP into a trichloroacetic acid-insoluble product The enzyme is solubilized with Triton X-100 from a nonnuclear particulate fraction and requires the participation of a heat-stable cofactor present in the same extract Its properties appear to be different from those of RNA polymerase and poly

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A or polyadenosine diphosphoribose synthetases The occurrence in brain of RNA polymerase[10–12] and homopolymer synthetase[13] has been described by several authors

MATERIALS AND METHODS

Enzyme assay

The incorporation of [^{14}C]ATP was followed at 30° in 0 2 ml of a solution containing 10 μmoles Tris–HCl pH 7 5, 0 3 μmole $MnCl_2$, 0 02 μmole [^{14}C]ATP (20–60 mC/mmole), 0 05 μmole GTP, 0 2 μmole mercaptoethanol, 100 μl of "cofactor" and 50 μl of enzyme which was added to start the reaction The incubation was terminated by addition of 2–3 volumes of 10% trichloroacetic acid After 10 min in ice, more 5% trichloroacetic acid was added, the precipitate was collected by centrifugation and washed 4 times with approx 10 ml of 5% trichloroacetic acid The washed precipitate was filtered through millipore with additional 5% trichloroacetic acid and counted on a low-background gas-flow counter Zero time controls (30–40 counts/min) were always subtracted The activity of the enzyme was expressed as pmoles of ATP incorporated In some experiments dealing with the characterization of the product (chromatography on Sephadex, sedimentation in sucrose density gradients, phenol and chloroform partition) [^{3}H]ATP obtained from the New England Nuclear Co was used as substrate after being diluted to a specific activity of 40 mC/mmole In this case the acid-washed product was dissolved in 0 4 ml hyamine and counted in an appropriate scintillator

Preparation of the enzyme

Step 1 Freshly excised rat brains (30–50 g), cleaned from membranes and blood vessels, were hand-homogenized in the cold in a Dounce glass homogenizer with 10 ml/g of tissue of 0 32 M sucrose containing 5 mM mercaptoethanol Centrifugations were carried out with the SS-34 rotor of the Sorvall RC-2 refrigerated centrifuge The particulate fraction obtained at 15 000 $\times$ *g* for 10 min (11 240 rev /min) was homogenized with an amount of 0 05 M Tris–HCl, pH 7 5, containing 5 mM mercaptoethanol corresponding to the original volume of the homogenate and recentrifuged as above The washed precipitate was homogenized in one volume of the same Tris–HCl buffer and brought to 0 5% Triton X-100 by addition of a 10% solution of the detergent The suspension was centrifuged at 105 000 $\times$ *g* for 90 min in rotor 40 of the Spinco ultracentrifuge (40 000 rev /min) The supernatant fraction was separated carefully from the pellet and the fluffy layer, and represented the Triton extract Under these conditions more than 80% of the enzyme was recovered in the supernatant phase A second extraction yielded only a minor fraction of the activity (approx 8%)

Step 2 5 ml ethanol were added slowly and with stirring to 10 ml of Triton extract at −5° The suspension was centrifuged for 5 min at 12 000 rev /min at approx −10°, the precipitate was dissolved in 1 8 ml of 0 05 M Tris–HCl pH 7 5, containing 5 mM mercaptoethanol and dialyzed in the cold for 6 h against 2 l of the same buffer Undissolved material was removed by centrifugation The enzyme preparation was stored at −15° with little or no loss of activity for at least one week

Preparation of the "cofactor"

10 ml of the Triton extract were heated at 80–90° for 15 min, cooled in ice and centrifuged at 15 000 rev /min for 10 min to remove the precipitate The supernatant fraction, which was stored at −15°, contained the cofactor but was devoid of enzyme activity

Materials

^{14}C-labeled ATP, ADP and GTP were from the New England Nuclear Co (Boston, Mass), [^{14}C]AMP from the Radiochemical Centre (Amersham, England) Triton X-100 was kindly given by Rohm and Haas Deoxyribonuclease, ribonuclease, venom phosphodiesterase and alkaline phosphatase from *Escherichia coli* were from Worthington Biochem Co (Freehold, New Jersey), phosphoenolpyruvate kinase from Boehringer (Mannheim, Germany), Actinomycin D was a gift of the Merck, Sharp and Dohme Co (Rahway, N J) Ribosomal RNA was prepared from brain ribosomes[14], soluble RNA was from *E coli* B (General Biochemistry) All other reagents were high grade commercial products

RESULTS

Solubilization of the enzyme

During the course of an investigation on the effect of nonionic detergents on the physical properties of RNA polymerase from rat brain, it was noted that the ATP-incorporating activity of crude nuclear fractions prepared from 0 32 M or 0 25 M sucrose homogenates (10 min at 1000 × *g* or 600 × *g* respectively) was considerably increased by addition of Triton X-100 The effect was dependent on the concentration of detergent and became maximal between 0 3 and 1% (Fig 1) At higher concentrations there was a progressive inhibition of the Triton-induced activity

Since non-ionic detergents produce a solubilization of membrane-bound proteins, it appeared likely that the enhanced activity was associated with the release of an enzyme in the soluble phase Such a possibility was tested by subjecting the

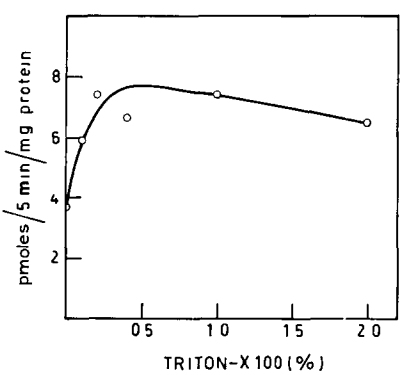


Fig 1 Effect of Triton X-100 on the ATP-incorporating activity of a crude nuclear fraction prepared by centrifugation at 600 × *g* for 10 min of a 20% homogenate of rat brain (0 25 M sucrose, 5 mM mercaptoethanol) The nuclear pellet was washed once by centrifugation and resuspended in 0 05 M Tris–HCl, pH 7 5 Assay medium as described in Table I, Expt 1

TABLE I

REQUIREMENTS OF THE REACTION

The results were consistently reproduced using the Triton X-100 extract as enzyme

Expt No	*Conditions*	*Activity (pmoles/ 5 min)*
1	Complete*	123
	− DNA	104
	− NaF	111
	− phosphoenolpyruvate, − phosphoenolpyruvate kinase	107
	− Mg^{2+}	122
	− Mn^{2+}	79
	+ $(NH_4)_2SO_4$, 0.05 M	66
2	Complete**	113
	+ deoxyribonuclease, 10 μg	102
3	Enzyme, 15 min at 18 **	134
	+ deoxyribonuclease, 10 μg	127
4	Complete**	95
	+ ribonuclease, 2 μg	84
	+ rRNA, 100 μg	93
	+ tRNA, 100 μg	86
5	Complete**	102
	− GTP	21
	− GTP, + UTP, 0.5 mM	71
	− GTP, + CTP, 0.5 mM	27
	+ UTP, + CTP	98
	− GTP, − UTP, + CTP	70
	+ P_i, 0.1 M	22

* Enzyme assays carried out in a medium designed to measure the activity of RNA polymerase and containing, in a volume of 0.2 ml, 36 μmoles Tris–HCl (pH 8.1), 1 μmole $MnCl_2$, 0.5 μmole $MgCl_2$, 5 μmole NaF, 0.5 μmole phosphoenolpyruvate, 4 μg phosphoenolpyruvate kinase 0.2 μmole mercaptoethanol, 25 μg DNA, 0.1 μmole each of GTP, CTP and UTP and 0.02 μmole [^{14}C]ATP (10–13 mC/mmole)

** Experiments carried out with the standard medium (see MATERIALS AND METHODS) containing however 36 μmoles Tris–HCl (pH 7.5) and 0.1 μmole GTP

Incubations were carried out at 37°

nuclear suspension treated with 0.5% Triton, 0.05 M Tris–HCl (pH 7.5) to centrifugation at 105 000 × *g* for 90 min This treatment yielded a supernatant fraction (Triton extract) which contained a sizeable part of the enzymic activity (approx 50%) and an insoluble residue whose activity was approximately equal to the amount present before Triton treatment The latter was attributed to the RNA polymerase present in the nuclei

Requirements of the reaction

The rate of ATP incorporation catalyzed by the Triton extract was linear for 10–15 min and rapidly declined afterwards In order to measure the initial velocity of the reaction, the assays were limited to 5 min or even shorter times As shown in Table I, the incorporation of ATP into acid-insoluble material was not dependent on

the presence of NaF or the energy-generating system (phosphoenolpyruvate and phosphoenolpyruvate kinase) and was markedly inhibited by $(NH_4)_2SO_4$ (Expt 1) and P_i (Expt 6) Omission of DNA, addition of deoxyribonuclease to the reaction mixture, or preincubation of the enzyme with deoxyribonuclease failed to produce any effect on the enzymic activity In other experiments carried out with a more purified preparation (ethanol precipitate, see *Preparation of the enzyme*) it was found that 2.3×10^{-5} M actinomycin D did not produce any inhibition The reaction was also essentially unaffected by addition of ribonuclease to the assay mixture and no stimulation was given by either soluble or ribosomal RNA

As to the ionic requirements, omission of Mn^{2+} produced a significant decrease in the rate of the reaction (Expt 1, Mg^{2+} present) but no effect was observed by lack of Mg^{2+} (Expt 1, Mn^{2+} present) When neither ion was added, approx 25% of the activity was still observed No additional stimulation was brought about by Mg^{2+} in presence of Mn^{2+}

A considerable decrease in the reaction rate was observed by omitting GTP (Expt 5) Under these conditions, addition of UTP produced a partial restoration of the activity while CTP was only slightly active The stimulatory effect of GTP did not require the presence of the other nucleoside triphosphates Similarly, CTP did not enhance the activation brought about by UTP It was concluded that the reaction did not require the simultaneous presence of the four nucleoside triphosphates, and that each nucleotide stimulated the enzyme activity to a different degree

Presence of a cofactor

The presence of an activating factor in the Triton extract was indicated by the anomalous behaviour of the curve relating enzyme activity to enzyme concentration, showing that the rate of the reaction was not proportional to the concentration of enzyme (Fig 2) Direct evidence for an activating component was provided by experiments in which the activity of the Triton extract was measured in presence of varying amounts of extract previously inactivated by heat (see *Preparation of the "cofactor"*) As shown in Fig 3, the rate of the reaction was stimulated severalfold by this preparation which in itself was completely devoid of enzyme activity

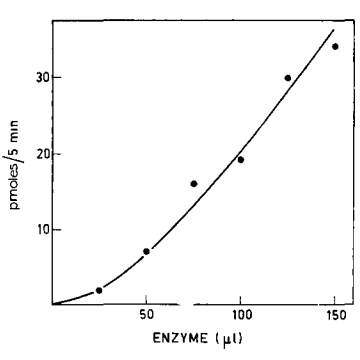


Fig 2 Dependence of the velocity of the reaction on the concentration of Triton X-100 extract

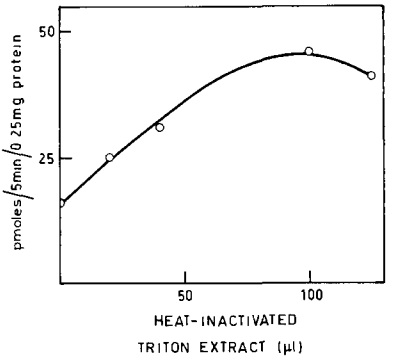


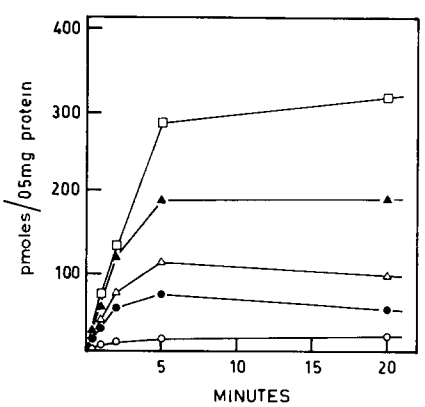
Fig 3 Effect of heat-inactivated Triton X-100 extract on the activity of the enzyme Assay carried out in the standard medium containing however 36 µmoles Tris–HCl (pH 7.5) and 0.1 µmole GTP

TABLE II

OUTLINE OF THE STEPS FOLLOWED IN THE PARTIAL PURIFICATION OF THE ENZYME

Fraction	*Total activity (pmoles/ 5 min)*	*Specific activity (pmoles/ 5 min per mg protein)*
Homogenate	90 000	69
Precipitate at 15 000 × *g* for 10 min	61 000	82
Spinco Supernatant	54 500	196
33% ethanol precipitate	39 100	425

Treatment of the Triton extract with ethanol at the final concentration of 33% (see *Preparation of the enzyme*) yielded a protein precipitate which contained most of the enzyme and essentially no cofactor and a soluble phase which retained most of the original cofactor activity Such a procedure produced a limited degree of purification, the specific activity of the ethanol precipitate being approx 6 times higher than that of the homogenate (Table II) With the ethanol precipitate there was little incorporation of [^{14}C]ATP in the absence of cofactor while addition of this component produced a stimulation of more than 20-fold in the rate of the reaction (from 16 to 400 pmoles/5 min per mg protein) Since the same amount of cofactor increased the activity of the Triton extract less than 3-fold, this finding confirmed the essential separation of the two components achieved by ethanol fractionation Analysis of the kinetics of ATP incorporation in presence of different concentrations of cofactor indicated that the amount of product formed was a function of the amount of cofactor and that also the initial rate of the reaction appeared to depend on the concentration of this component (Fig 4)



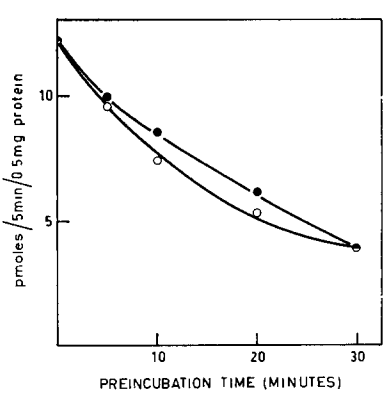
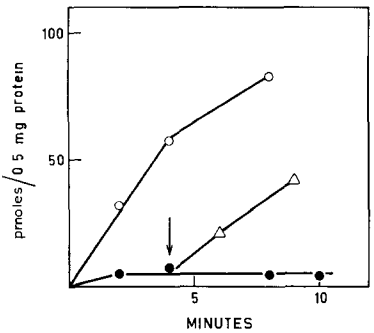


Fig 4 Effect of the cofactor concentration on the kinetics of the reaction [α-^{32}P]ATP (23 8 mC/ mmole) was used as substrate in the standard assay ○, cofactor omitted, ●, 20 μl cofactor, △, 40 μl cofactor, ▲, 80 μl cofactor, □, 100 μl cofactor

Fig 5 Kinetics of inactivation of the enzyme Two samples of the ethanol precipitate were incubated at 30° with (●) and without (○) cofactor, added in the same proportion as in the assay medium Aliquots were taken at the desired time intervals and assayed under the standard conditions



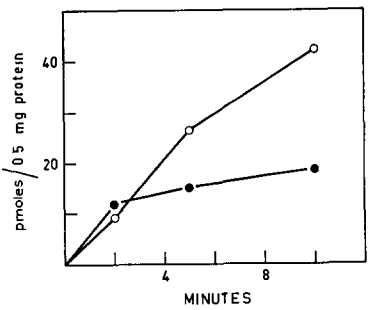


Fig 6 Addition of the cofactor during the course of the reaction Cofactor present from the beginning in a standard assay (○), cofactor replaced by an equal volume of water (●), cofactor added after 4 min (△) The slightly lower rate observed under the latter conditions is due to the dilution of the sample

Fig 7 Apparent loss of cofactor after incubation with the enzyme Two complete sets of standard mixtures were incubated at 30° for 2 min and 10 min, respectively, and treated thereafter at 80–90° for 10 min Aliquots of 0 2 ml were then incubated with 0 05 ml of fresh enzyme at 30° for 2, 5 and 10 min ○, preincubated for 2 min, ●, preincubated for 10 min

The possibility that the role of the cofactor was that of preventing a rapid inactivation of the enzyme was considered unlikely in view of this last effect and was excluded by the finding that the rate of enzyme inactivation at 30° was unaffected by addition of this component and was far too slow to account for the drastic loss of activity observed when the cofactor was omitted (Fig 5) It was also found that the rate of a reaction carried out without added cofactor, which had stopped after the first minutes of incubation, was fully restored by addition of the heat-stable component (Fig 6) This finding indicated that the limited extent to which the reaction occurred under these conditions was due only to lack of cofactor and not to the inactivation of the enzyme, and raised the possibility that the heat-stable component was being consumed during the process of enzymic catalysis Some support for this possibility was obtained by the results presented in Fig 7 When the cofactor was incubated for 2 min with the enzyme under the standard conditions and the mixture was then treated at 80–90° (a procedure known to inactivate the enzyme but to preserve the cofactor and the other components of the assay mixture), addition of fresh enzyme elicited a rate of ATP incorporation which remained approximately linear for 10 min On the other hand, when the preincubation of enzyme and cofactor lasted for a longer time (10 min), the rate of the subsequent reaction stopped after the first few minutes In addition, if the preincubation was carried out for 10 min but without ATP in order to prevent the occurrence of the enzymic reaction, the high rate obtained after heating the mixture and adding the substrate indicated that no cofactor had been lost under these conditions

Properties of the cofactor

The suggestion that the cofactor was a molecule of relatively small molecular weight, provided by its heat-stability, was confirmed by the finding of its dialyzability through cellulose bags (Table III) Assay of the lyophylized outer solution after dialysis afforded an almost complete recovery of the cofactor activity

TABLE III

DIALYZABILITY OF THE COFACTOR

An aliquot of the cofactor preparation (2 ml) was dialyzed at 4° against 2 l of 10 mM Tris–HCl (pH 7.5) with continuous stirring of the outside solution. Storage of the cofactor at the same temperature and for the same period of time did not affect its activity.

Additions	*Activity (counts/min per 5 min)*
None	334
Cofactor	886
Cofactor, dialyzed 48 h	224
Cofactor, dialyzed 90 h	321

Treatment of the cofactor at 20° for 15 min with deoxyribonuclease (100 μg/ml) or ribonuclease (20 μg/ml) did not decrease its stimulatory activity. On the other hand, incubation with alkaline phosphatase produced a complete loss of its capacity to enhance the rate of the reaction (Table IV). No inactivation occurred when the incubation was carried out under the same conditions but in the absence of alkaline phosphatase. No effect on the ATP incorporation was produced by equivalent volumes

TABLE IV

EFFECT OF HYDROLYTIC ENZYMES ON THE COFACTOR ACTIVITY

Incubation of the cofactor was carried out at 37° for 4 h. The medium for alkaline phosphatase contained 10 mM Tris–HCl, pH 9.0. After incubation the mixtures were neutralized, heated at 80–90° for 30 min to destroy the hydrolytic enzymes and equivalent aliquots were assayed for cofactor activity using reaction mixtures lacking this component (5-min incubation).

Additions	*Activity (counts/min)*
None	267
Cofactor	704
Cofactor, 4 h at 37°	690
Cofactor, 4 h at 37°, alkaline phosphatase (20 μg/ml)	272

of incubation mixtures containing alkaline phosphatase but lacking the cofactor. These data suggested the presence of a phosphate group required for the activity of the cofactor.

Properties of the product

A marked instability of the product at neutral or moderately alkaline conditions was shown by incubating it at different pH values (Fig. 8). Loss of trichloroacetic acid-insoluble material occurred above pH 6, increasing as the pH increased. At pH 8.0 approximately half of the product became acid-soluble after 3 h of incubation at 30°. In view of this instability, experiments dealing with the possible effects of hydrolytic enzymes on the product were carried out at pH 5.5 (Table V). Under these conditions, even prolonged incubation with deoxyribonuclease or ribonuclease failed

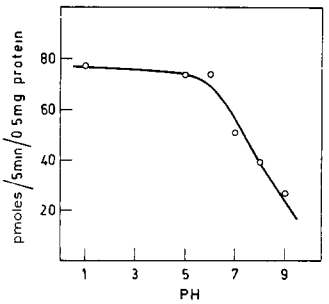


Fig 8 Effect of pH on the degradation of the reaction product at 30 Aliquots of the acid-washed product were incubated at the desired pH values for 3 h and reprecipitated with trichloroacetic acid

to release any acid-soluble material A significant degradation was however produced by phosphodiesterase which rendered approx 37% of the product acid-soluble in 2 h Essentially the same extent of degradation (43%) was brought about by doubling the time of incubation and by adding an additional aliquot of phosphodiesterase

Several proteolytic enzymes (Table V) did not affect the precipitability of the product in acid In addition, no effect was observed after incubation at 30° for 3 h with pepsin (50 μg) and with trypsin *plus* chymotrypsin (100 μg each) It was however found that half of the pronase-treated product became non-sedimentable at 17 000 $\times$ *g* for 10 min, while without pronase approx 90% of the trichloroacetic acid-washed product was recovered in the sediment A considerable decrease in the opacity of the product occurred during its incubation with pronase

Addition of 1% sodium dodecylsulfate to the suspension of product in 0 05 M

TABLE V

EFFECT OF HYDROLYTIC ENZYMES ON THE REACTION PRODUCT

The product of the reaction was washed 3 times with 5% trichloroacetic acid and incubated at 30 in volumes of 0 5 ml in 0 05 M sodium acetate, pH 5 5 Ribonuclease (20 μg) deoxyribonuclease (200 μg), phosphodiesterase (500 μg) and $MgCl_2$ (1 μmole), pronase (500 μg), streptolysine (500 μg), papain (270 μg), cysteine (1 μmole) and EDTA (0 5 μmole) In Expt 2 in which [^{3}H]-ATP (40 mC/mmole) was used as substrate, the product was solubilized in 0 5 ml hyamine and counted in a toluene scintillator All the enzymes tested were active on their respective substrates under the conditions used Appropriate controls run when the incubation mixture contained additional components were not different from the normal one Results are expressed as counts/min of trichloroacetic acidprecipitable product

Experiment	*Additions*	*Incubation time*	
		120 min	*240 min*
1	Control	2100	1835
	Ribonuclease	2050	1800
	Deoxyribonuclease	2100	1870
	Phosphodiesterase	1320	1025
2	Control	3650	
	Pronase	3500	
	Streptolysine	3690	
	Papain	3700	

sodium acetate buffer (pH 5 5) produced a complete clarification Shaking 0 5 ml of this preparation with an equal volume of water-saturated phenol at room temperature for 15 min afforded a 40% recovery of the trichloroacetic acid-insoluble radioactivity in the aqueous phase while the remaining radioactivity remained associated with the phenol layer A similar distribution was obtained when the partition was carried out with chloroform A slightly higher recovery in the aqueous phase (45%) was provided by phenol treatment of the product not previously treated with trichloroacetic acid, with or without addition of 1% sodium dodecylsulfate

Molecular sieving on Sephadex G-100 of the acid-washed product dissolved in 0 05 M sodium acetate buffer containing 1% sodium dodecylsulfate after equilibration of the column (1 cm × 25 cm) with the same solvent yielded a radioactive peak after 9 ml Under the same conditions, a preparation of tRNA from *E coli* was eluted after 11 ml, while the peak of ATP appeared after 26 ml Similar results were obtained with a column of Sephadex G-25 with which the elution volumes for ATP, tRNA and the product were 24, 12 and 7 ml, respectively In the absence of sodiumdodecylsulfate the elution of tRNA on Sephadex G-100 was not different from that obtained in presence of the detergent, while those of two proteins run as standards were significantly increased (from 11 to 18 ml in the case of cytochrome *c*, from 9 to 12 ml in the case of bovine serum albumin)

Less conclusive findings were provided by centrifugation of a mixture of product and tRNA in a 3–12% sucrose density gradient containing 0 05 M sodium acetate buffer (pH 5 5) and 1% sodium dodecylsulfate (20 h at 24 000 rev /min, 15°) The percentage distribution of radioactivity indicated the presence of product in the top region of the 16-ml gradient with a leading edge extending 4–5 ml from the top, while tRNA sedimented with a peak at 3 ml from the top The resolution achieved was not sufficient to allow discrimination in the region between tRNA and more slowly sedimenting compounds

After alkaline hydrolysis of the product in 0 3 M KOH (18 h at 37°), only a

TABLE VI

CHROMATOGRAPHY OF THE ALKALINE-HYDROLYZED PRODUCT ON DEAE CELLULOSE

Percentage distribution of the fragments obtained by alkaline hydrolysis of the product separated by thin-layer chromatography on DEAE-cellulose[15] The concentration of HCl refers to the eluting solution The reference compounds visualized under ultraviolet light and the blank regions in between were counted in a Tricarb scintillation counter Recoveries approached 90%

Standard	*HCl conen*	
	$3\ 10^{-2}\ M$	$2\ 10^{-1}\ M$
Adenosine	8 0	
Blank	3 3	
Adenosine-5′-monophosphate	11 0	56 0
Blank	2 3	
Adenosine-5′-diphosphate	28 0	
Blank		14 0
Adenosine-5′-triphosphate		9 7
Blank	47 0	5 9
Adenosine-5′-tetraphosphate		11 2
Blank		1 0

portion of the radioactivity was recovered with the adenosine, AMP or ADP standards from columns of Dowex 1 (formate) and from thin-layer chromatograms (Table VI) The remaining radioactivity was either not eluted from columns of Dowex 1 (formate) under the conditions used (4 M formic acid or moved as more highly-charged negative components when subjected to thin-layer chromatography (Table VI) These compounds were not readily identified with the phosphorylated derivatives of adenosine used as standards (ATP and adenosine tetraphosphate) since blank regions of the chromatogram had a comparatively high content of radioactivity

Other substrates

Under the standard assay conditions but without GTP in the reaction mixture, ADP was incorporated into acid-insoluble material at approximately one-third the rate of ATP (Table VII) Under similar conditions, the incorporation of AMP was

TABLE VII

INCORPORATION OF OTHER SUBSTRATES

The assays were carried out under the standard conditions using the ethanol precipitate as the enzyme, except that GTP was omitted Each substrate was present in the concentration of 1 10^{-4} M

Addition	*Activity (pmoles/ 5 min)*
[^{14}C]ATP	50
[^{14}C]ATP, NMN (2 10^{-3} M)	47
[^{14}C]ATP, NAD (2 10^{-3} M)	44
[^{14}C]ADP	17
[^{14}C]ADP, ATP (1 10^{-3} M)	9
[^{14}C]ADP, glucose (0 1 M), hexokinase (80 μg)	0
[^{14}C]AMP	2
[^{14}C]GTP	0

minimal and was completed after the first minute of incubation The velocity of ADP incorporation was markedly decreased by addition of a 10-fold excess of ATP and was brought to insignificant values in the presence of an ATP-trapping system (glucose and hexokinase) It appeared therefore that the incorporation of adenosine diphosphate followed the previous conversion of this compound into [^{14}C]ATP catalyzed by the same enzyme preparation This was confirmed by the finding that a considerable portion of [^{14}C]ADP chromatographed as ATP on thin layers of DEAE-cellulose[15] after incubation with the enzyme for 10 min under the standard assay conditions

Because of its marked activating effect, GTP was also examined as a possible substrate of the enzyme, but no incorporation was found (Table VII) In the same table it is also reported that NMN and NAD have no effect on the incorporation of [^{14}C]ATP This property differentiates the enzyme from the adenosine diphosphoribose polymerase of liver which is strikingly stimulated by NMN[16] and whose activity is considerably decreased by addition of unlabeled NAD[7,8] which dilutes the true substrate of the enzyme

TABLE VIII

DISTRIBUTION OF THE TRITON X-100 EXTRACTED ENZYME ACTIVITY IN SUBCELLULAR FRACTIONS FROM RAT BRAIN

Enzyme activity was measured in Triton X-100 extracts prepared from each of the particulate fractions obtained by differential centrifugation of a 10% homogenate of rat brain in 0.32 M sucrose

Fraction	*Total activity (pmoles/5 min)*	*%*	*Specific activity (pmoles/5 min per mg protein)*
1 000 × *g*, 10 min	7 900	12.3	250
15 000 × *g*, 10 min	45 100	71.0	342
35 000 × *g*, 10 min	5 550	8.7	250
105 000 × *g*, 60 min	5 100	8.0	76

Distribution of the enzyme

Only a small percentage of the total ATP-incorporating activity present in a 10% homogenate of rat brain was recovered in the precipitate collected at 1000 × *g* for 10 min (nuclear fraction), whereas most of the enzyme was found associated with the particulate sedimenting at 15 000 *g* for 10 min (Table VIII). The highest value of specific activity was also exhibited by this fraction. Some enzyme was extracted from the fractions sedimenting at higher velocities (up to 105 000 *g* for 60 min), but no activity was detected in the Spinco supernate even after Triton X-100 treatment.

The finding that only a small portion of the enzyme was extracted from the nuclear fraction suggested a different subcellular localization. This was confirmed in an experiment in which the crude nuclear fraction was fractionated into a pellet of purified nuclei and a floating layer containing nuclei and other nonnuclear particulates. As shown in Table IX, the purified nuclei contained some activity which was not stimulated by Triton X-100, whereas the floating layer catalyzed the incorporation of

TABLE IX

LACK OF TRITON X-100 INDUCED ACTIVITY IN PURIFIED NUCLEI

A 10% homogenate of rat brain in 0.32 M sucrose was centrifuged at 1000 *g* for 10 min to collect the crude nuclear fraction which was washed once by centrifugation in the same medium. The final pellet was resuspended with approx. 7 vol. of 2.3 M sucrose, 5 mM mercaptoethanol and centrifuged in rotor SW-25 of the Spinco ultracentrifuge at 23 000 rev./min for 2 h. The purified nuclear pellet and the heterogeneous layer floating on top of the 2.3 M sucrose were resuspended in 0.05 M Tris–HCl (pH 8.3) and divided into two aliquots, one of which received Triton X-100 to the final concentration of 0.5%. The enzyme activity was measured by the incorporation of ATP under conditions that would also detect the activity of RNA polymerase (see Table I, Expt. 1).

Fraction	*Total activity (pmoles/5 min)*		
	Without Triton X-100	*With Triton X-100*	*Triton X-100 induced*
Purified nuclei	84	70	0
Floating layer	420	1520	1100

ATP in a reaction which was markedly stimulated by Triton X-100. The incorporation of ATP in the absence of detergent was attributed to the activity of the nuclear RNA polymerase. Triton X-100-induced activity was therefore associated with the fraction containing non-nuclear particulate material but absent in the purified nuclear fraction. In accord with this result, a Triton X-100 extract prepared from the purified nuclear pellet contained no enzyme activity.

A similar ATP-incorporating activity was also present in other organs of the rat where it was identified by the solubilizing effect of Triton X-100 on similarly prepared particulate fractions (15 000 × *g* for 10 min) and by the activating effect of the cofactor. A comparable level of enzyme was found in brain, liver and heart, while considerably lower amounts (approx. 5%) were detected in kidney and spleen.

Discussion

Since the formation of acid-insoluble products from ATP in mammalian tissues is generally attributed to the catalytic action of RNA polymerase and poly A synthetase, respectively, the ATP-incorporating activity of rat brain described in this paper should be primarily compared with these two enzymes. The main properties which differentiate it from RNA polymerase are (1) the lack of a DNA requirement, (2) the capacity to attain full activity with only ATP, (3) the lack of incorporation of GTP, (4) the resistance of the product to ribonuclease, (5) the nature of the fragments obtained by alkaline hydrolysis of the product, (6) the non-nuclear localization. The enzyme can also be readily distinguished from poly A synthetase by virtue of the following properties: (1) the nature of the product formed, (2) the activation by GTP, (3) the lack of requirement for an RNA primer, (4) the limited time course of the reaction. A third polynucleotide-synthesizing enzyme which appears to be different from the ATP-incorporating activity described in this paper is the poly adenosine diphosphoribose synthetase described in liver nuclei[7–9,16] as shown by the lack of effect of NAD and NMN. In the reaction catalyzed by polyadenosine diphosphoribose synthetase, NAD serves as the substrate and NMN is an absolute requirement for the incorporation of radioactivity from ATP into the product.

As to the identity of the product of the reaction, we have listed above the main reasons which appear to exclude a similarity with RNA or poly A. An additional property which distinguishes it from these two polymers is its striking sensitivity to moderately alkaline pH conditions. This property argues also against the possibility of a protein adenylylation similar to that described for glutamine synthetase from *E. coli*[17] in which case the adenylate is not released after alkaline hydrolysis at 37° (0.3 M NaOH for 3 h). The similar chromatographic behaviour of the product and tRNA on Sephadex G-25 and G-100, while suggestive of an association of the incorporated radioactivity with a compound of considerable molecular weight, should be viewed with caution because of the significant effect of sodium dodecylsulfate on the elution volume of proteins. Despite the permanence of the product as acid-insoluble material after digestion with several proteolytic enzymes, its association with a protein cannot be excluded, particularly since it is only partial recovered in the aqueous phase after phenol or chloroform extraction and also in view of the effect of pronase and sodium dodecylsulfate on its solubility under neutral conditions. The partial hydrolysis of the product by phosphodiesterase from snake venom indicates the pre-

sence of phosphodiester bonds, but suggests also the occurrence of a resistant residue still precipitable with trichloroacetic acid

Two other features which appear to be peculiar to this enzyme are the striking activating effect of GTP and, to a lesser extent, of UTP and CTP, and the strict requirement of the reaction for a heat-stable cofactor present in the same enzyme extract It should be stressed that the activation brought about by the nucleoside triphosphates does not require the simultaneous presence of all the four nucleotides and is not accompanied by the incorporation of the activator, as shown in the case of GTP The heat-stable cofactor appears to be of relatively low molecular weight, as shown by its dialyzability through cellulose bags The presence of an esterified phosphate group is suggested by the loss of stimulatory activity produced by alkaline phosphatase It should be noted that a mixture of 2′, 3′-AMP, GMP, UMP and CMP (1 mM each) was unable to replace the cofactor Although the loss of cofactor activity which occurs during enzymatic incubation could be due to the action of unspecific reactions, the finding that no such loss takes place when the incubation is carried out without substrate suggests a more specific mechanism involving perhaps the incorporation of the cofactor in the reaction product In such a case, elucidation of the nature of the product would be greatly eased by a previous understanding of the nature of the cofactor

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