Effect of 2-Allyl-2-isopropylacetamide on Poly(Adenylic Acid)-Containing Ribonucleic Acid

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(Received 11 February 1976)

A single administration of 2-allyl-2-isopropylacetamide, a porphyrinogenic drug, enhanced the $^{32}$P-labelling of nucleoplasmic as well as cytoplasmic poly(A)-containing RNA in rat liver. The synthesis of total microsomal RNA is only marginally increased under these conditions. The drug enhances the labelling of a variety of cytoplasmic poly(A)-containing RNA species, and this effect is counteracted by the simultaneous administration of haemin. 2-Allyl-2-isopropylacetamide also enhanced the release of RNA from the nucleus to the cytoplasm.

2-Allyl-2-isopropylacetamide is a powerful inducer of δ-aminolaevulinate synthetase, the first and rate-limiting enzyme of the haem-biosynthetic pathway (Granick, 1966). Haem has been implicated as a regulator of this enzyme induction (Granick, 1966; De Matteis 1971; Padmanaban et al., 1973). On the basis of susceptibility of the induction process to inhibitors of nucleic acid synthesis, it has been suggested that RNA synthesis mediates the effects observed (Granick, 1966; Sassa & Granick, 1970). It has been shown that 2-allyl-2-isopropylacetamide increases nucleoplasmic RNA synthesis in rat liver (Sardana et al., 1975). Nawata & Kato (1973) have reported that 3,5-diethoxycarbonyl-1,4-dihydrocololidine, another potent inducer of δ-aminolaevulinate synthetase, increases total nuclear RNA synthesis in rat liver. Incely et al. (1974) have reported the increase in nucleoplasmic RNA polymerase activity in chick-embryo liver cell cultures exposed to 2-allyl-2-isopropylacetamide.

The present studies reveal that 2-allyl-2-isopropylacetamide enhances the labelling of cytoplasmic poly(A)-containing RNA associated with microsomal/polyribosomal fractions by activating the transcription process as well as the transport of the RNA from the nucleus to the cytoplasm. On the basis of the fact that most euakaryotic mRNA has poly(A) at the 3'-end (Brawerman, 1974), it is concluded that 2-allyl-2-isopropylacetamide increases the amounts of possibly several mRNA species and that this early effect of 2-allyl-2-isopropylacetamide is counteracted by haemin.

Materials and Methods

Male abino rats (110–120g) of the Indian Institute of Science strain were used in these experiments. $^{32}$P (carrier-free) was purchased from the Bhabha Atomic Research Centre, Trombay, India. 2-Allyl-2-isopropylacetamide was generously supplied by Hoffmann–La Roche, Basle, Switzerland. Poly(U)–Sepharose was a gift from Professor M. Rabinowitz, University of Chicago, Chicago, IL, U.S.A.

Treatment of animals

The animals were starved for 24h and then injected with 2-allyl-2-isopropylacetamide (400mg/kg) subcutaneously. At 30min after 2-allyl-2-isopropylacetamide administration, $^{32}$P was injected intraperitoneally; the animals were killed at different time-intervals and the livers processed.

$^{32}$P incorporation into nucleoplasmic and nucleolar RNA

For this purpose nuclei were isolated from pooled livers by the method of Blobel & Potter (1966). Nucleolar and nucleoplasmic fractions were isolated by the procedure described by Muramatsu & Fujisawa (1968). The procedures for RNA extraction was as described by Abe et al. (1972) and measurement of the specific radioactivity of the nucleotide pool as described by Sardana et al. (1975).

$^{32}$P incorporation into poly(A)-containing RNA

The animals were killed 4h after $^{32}$P administration and the livers were homogenized in 0.25M-sucrose containing 10mM-Tris (pH7.4) and polyvinyl sulphate (20µg/ml) and heparin (100µg/ml). The microsomal fraction was isolated from the postmitochondrial supernatant by spinning at 10000g for 60min. Cytoplasmic polyribosomes were isolated from the postmitochondrial supernatant by using the magnesium-precipitation method described by Georgiev et al. (1972) or by centrifugation of deoxycholate-treated microsomal preparations at 105000g for 2h. RNA was extracted with phenol/
chloroform/3-methylbutan-1-ol as described by Penman (1966). Poly(A)-containing RNA was isolated by using nitrocellulose filters (Brawerman et al., 1972) as well as by poly(U)-Sepharose chromatography (Lindberg & Persson, 1972; Firtel et al., 1972). For the latter procedure, a 3 ml column of poly(U)-Sepharose, equilibrated with high-salt buffer (400 mm - NaCl / 2 mm - EDTA / 10 mm - Tris, pH 7.5 and 0.2% sodium dodecyl sulphate), was used. The labelled RNA preparation from microsomal or polyribosomal fractions was solubilized in high-salt buffer, and 1–1.5 ml of the sample containing 20–30 E260 units was then loaded on the column at 25°C. After 5 min, the column was washed with 9 vol. of high-salt buffer and the poly(A)-containing RNA was finally eluted with 4 ml of formamide buffer [90% (w/v) formamide, 2 mm-EDTA and 10 mm-potassium phosphate buffer, pH 7.5]. Yeast RNA (200 μg) was added as carrier to the eluate and the RNA was precipitated by the addition of 2.5 vol. of ethanol. The final preparation obtained was subjected to a second cycle of poly(U)-Sepharose chromatography before it was used for polyacrylamide-gel-electrophoretic analysis and radioactivity measurements. Gel electrophoresis was carried out with 2.7% (w/v) gels in ‘E’ buffer (Loening, 1969). The gels were then sliced after freezing or after drying on filter papers in an oven and the slices were used for radioactivity measurements.

**Transport of RNA from the nucleus to the cytoplasm**

Control as well as 2-allyl-2-isopropylacetamide-treated rats were injected with 32P, and after 4 h the animals were killed. Purified nuclei (5 x 106/ml) from the livers were then incubated in a medium containing 50 mm-Tris/HCl (pH 7.5), 25 mm-KCl, 2.5 mm-MgCl2, 0.5 mm-CaCl2, 0.3 mm-MnCl2, 5.0 mm-NaCl, 2.5 mm-Na2HPO4, 5.0 mm-spermidine, 2.0 mm-dithiothreitol, 2.0 mm-ATP, 2.5 mm-phosphoenolpyruvate, 35 units of pyruvate kinase, 500 μg of yeast RNA per ml and dialysed cytosol (postmicrosomal supernatant). Additional phosphoenolpyruvate (1.0 mm) was added after each 10 min interval of incubation at 30°C. Dialysed cytosol was prepared separately both from control and 2-allyl-2-isopropylacetamide-injected animals. The entire procedure is based on the reports of Webb and co-workers (Schumm et Webb, 1972; Yu et al., 1972; Schumm et al., 1973). Samples were removed at different time-intervals, spun down to remove the nuclei and RNA was extracted from the supernatant with phenol/chloroform/3-methylbutan-1-ol (Penman, 1966). Incubation mixtures maintained at 0°C and processed similarly served as controls. The radioactivity in the RNA thus isolated was also measured before and after poly(U)-Sepharose chromatography.

**Results**

The effect of 2-allyl-2-isopropylacetamide on nucleoplasmic and nucleolar RNA synthesis as a function of time was first examined. The results presented in Fig. 1 indicate that 2-allyl-2-isopropylacetamide enhances 32P incorporation into nucleoplasmic RNA. The drug inhibits 32P incorporation into nucleolar RNA at early periods of labelling and drug treatment. These results are also borne out by the data presented in Table 1 where the results are expressed as a function of the specific radioactivity of the nucleotide pool.

Next it was examined whether the increase in nucleoplasmic RNA synthesis due to 2-allyl-2-isopropylacetamide treatment is also reflected in cytoplasmic RNA synthesis. For this purpose, RNA was isolated from the microsomal fraction of the livers of normal and drug-treated animals after a 4 h period of labelling with 32P. Labelling of poly(A)-containing RNA was also assayed, Millipore filtration and poly(U)-Sepharose chromatography being the techniques used to isolate this RNA fraction from the microsomal fraction as well as from the polyribosomes. In view of the earlier finding that haemin counteracts the enhanced nucleoplasmic RNA synthesis due to 2-allyl-2-isopropylacetamide treat-
ment (Sardana et al., 1975), the effect of haemin on 2-allyl-2-isopropylacetamide-mediated changes in cytoplasmic RNA synthesis was also examined. The results presented in Table 2 indicate that 2-allyl-2-isopropylacetamide increases labelling of total microsomal RNA only to a marginal extent (8–10%). The drug, however, increases the labelling of poly(A)-containing RNA fractions isolated by both the Millipore technique and poly(U)-Sepharose chromatography from microsomal as well as from polyribosomal RNA. It is significant to observe that haemin is able to counteract the enhanced labelling of poly(A)-containing RNA due to 2-allyl-2-isopropylacetamide treatment.

It was decided to examine whether the transport of RNA from the nucleus to the cytoplasm is affected under conditions of 2-allyl-2-isopropylacetamide treatment. For this purpose, pre-labelled nuclei from normal and drug-treated animals were incubated with non-radioactive cytosol prepared from both normal and drug-treated animals, as described by Webb and co-workers (Schumm & Webb, 1972; Yu et al., 1972; Schumm et al., 1973). The results presented in Fig. 2 indicate that there is enhanced transport of RNA from the nucleus to the cytoplasm under conditions of 2-allyl-2-isopropylacetamide treatment. Maximum release of RNA is observed under conditions where 32P-labelled nuclei from 2-allyl-2-isopropylacetamide-treated animals were incubated with the autologous cytosol. Assay of poly(A)-containing RNA species in the released RNA reveals (Table 3) that 2-allyl-2-isopropylacetamide treatment stimulates the release of this RNA fraction as well. Polyacrylamide-gel-electrophoretic analysis of released poly(A)-containing RNA in vitro from normal and 2-allyl-2-isopropylacetamide-treated animals is presented in Fig. 3. The bulk of the released poly(A)-containing RNA from normal animals moves to a position around 10S. Under conditions of 2-allyl-2-isopropylacetamide

Table 1. Effect of 2-allyl-2-isopropylacetamide on 32P incorporation into nucleoplasmic and nucleolar RNA of rat liver as a function of the specific radioactivity of the nucleotide pool

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nucleoplasmic RNA</th>
<th>Nucleolar RNA</th>
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<tbody>
<tr>
<td></td>
<td>10×</td>
<td>10×</td>
</tr>
<tr>
<td></td>
<td>Nucleotide pool</td>
<td>Nucleotide pool</td>
</tr>
<tr>
<td>Control</td>
<td>30min 2h 4h 6h</td>
<td>30min 2h 4h 6h</td>
</tr>
<tr>
<td>Allylisopropylacetamide</td>
<td>2.2 4.2 4.2 4.0</td>
<td>1.3 3.0 3.5 4.0</td>
</tr>
</tbody>
</table>

Table 2. Effect of 2-allyl-2-isopropylacetamide on 32P incorporation into microsomal RNA and poly(A)-containing RNA

32P (2.5 mCi/animal) was injected into the animals 30 min after the administration of 2-allyl-2-isopropylacetamide or 2-allyl-2-isopropylacetamide+haemin. The animals were killed 4 h after the administration of label and the livers were processed for the isolation of microsomal RNA, polyribosomal RNA and poly(A)-containing RNA as described in the Materials and Methods section. Column I refers to the results with poly(A)-containing RNA obtained from total microsomal RNA after two cycles of poly(U)-Sepharose chromatography. Column II refers to poly(A)-containing RNA from microsomal RNA by the Millipore technique. Column III refers to poly(A)-containing RNA from polyribosomal RNA by the poly(U)-Sepharose technique. As a routine 30 E260 units of 32P-labelled microsomal RNA was processed for poly(A)-containing RNA isolation. The results are expressed as c.p.m./E260 unit of RNA processed. The results presented are those obtained in a typical experiment. The values given in parentheses refer to the percentage of total RNA radioactivity in poly(A)-containing RNA, and the range indicated is obtained from four different experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Microsomal RNA</th>
<th>Poly(A)-containing RNA (c.p.m./E260 unit of RNA processed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m./E260 unit</td>
<td>10× Microsomal RNA Nucleotide pool</td>
</tr>
<tr>
<td>Control</td>
<td>11586</td>
<td>1.90</td>
</tr>
<tr>
<td>Allylisopropylacetamide</td>
<td>12702</td>
<td>2.10</td>
</tr>
<tr>
<td>Allylisopropylacetamide + haemin</td>
<td>11725</td>
<td>1.85</td>
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</table>
The animals were injected with $^{32}$P (2mCi/animal), 30 min after 2-allyl-2-isopropylacetamide or saline (0.9% NaCl) injection. The animals were killed 4 hr after injection of the label and nuclei prepared. Purified nuclei (5 x $10^8$/ml) were incubated in a total volume of 5 ml with cytosol obtained from control or 2-allyl-2-isopropylacetamide-treated animals and other components as described in the text. Portions (1.5 ml) were removed at three different time-intervals, centrifuged to remove the nuclei, and RNA was isolated from the supernatant. Samples maintained at 0°C served as controls for each time-point and the labelled RNA released at 0°C was found to be around 15–18% of the RNA released at 37°C. The results are expressed as the percentage of RNA released as a function of the total [32P]RNA present in the nuclei. Control nuclei contained 42,890 c.p.m./5 x $10^6$ nuclei. The corresponding value for nuclei isolated from 2-allyl-2-isopropylacetamide-treated animals was 47,970 c.p.m. ○, Nuclei(1)+cytosol(1); △, nuclei(1)+cytosol(1); ●, nuclei(II)+cytosol(II); ♦, nuclei(II)+cytosol(II). (1), Isolated from control animals and (II) isolated from 2-allyl-2-isopropylacetamide-treated animals.

Table 3. Effect of 2-allyl-2-isopropylacetamide in vivo on the release of poly(A)-containing RNA from nuclei in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount of RNA processed (c.p.m.)</th>
<th>Poly(A)-containing RNA (c.p.m.)</th>
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</thead>
<tbody>
<tr>
<td>Nuclei+cytosol*</td>
<td>5000</td>
<td>715</td>
</tr>
<tr>
<td>Nuclei+cytosol†</td>
<td>7200</td>
<td>1296</td>
</tr>
</tbody>
</table>

* Nuclei and cytosol isolated from control animals.
† Nuclei and cytosol isolated from 2-allyl-2-isopropylacetamide-treated animals.

The experimental conditions are described in Fig. 2. The entire amount of $^{32}$P-labelled RNA released during the first 10 min period was subjected to poly(U)-Sepharose chromatography and the poly(A)-containing RNA was isolated as described in the text.

The experimental details are as described in Fig. 2, except that the incubation was restricted to a period of 10 min. The entire RNA released during this period was isolated by phenol/chloroform/3-methylbutan-1-ol extraction, and the poly(A)-containing RNA was isolated by two cycles of poly(U)-Sepharose chromatography. The total amount of released RNA was: nuclei(I)+cytosol(II), 18,250 c.p.m.; nuclei(II)+cytosol(II), 23,985 c.p.m. The amounts of poly(A)-containing RNA recovered and subjected to electrophoretic analysis were: nuclei(I)+cytosol(II), 2520 c.p.m.; nuclei(II)+cytosol(II), 4250 c.p.m. Slices (2 mm) were prepared from gels and processed for radioactivity measurements. (1), Isolated from control animals and (II) isolated from 2-allyl-2-isopropylacetamide-treated animals. ○, RNA released from control nuclei in the presence of control cytosol; ●, RNA released from nuclei of 2-allyl-2-isopropylacetamide-treated animals in the presence of cytosol from drug-treated animals.

10S. Simultaneous haemin treatment results in a suppression of the enhanced labelling of poly(A)-containing RNA species in both the regions.
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microsomal RNA and cytoplasmic poly(A)-containing RNA. The marginal increase in microsomal RNA labelling due to 2-allyl-2-isopropylacetamide treatment is almost entirely due to poly(A)-containing RNA. In an earlier study, Nawata & Kato (1973) could not detect a significant change in the labelling of total polyribosomal RNA in rats injected with 3,5-diethoxycarbonyl-1,4-dihydrocollidine.

The studies also reveal that the increased labelling of poly(A)-containing RNA is due to both enhanced transcription and transport from the nucleus. It is interesting to observe that the cytosol prepared from 2-allyl-2-isopropylacetamide-treated animals is more potent than that from normal animals in the release of RNA from the nucleus. The importance of factors present in the cytosol for the release of RNA from the nucleus has also been stressed by Webb and co-workers (Schumm & Webb 1972, 1974). Schumm & Webb (1974) and Shchuppe et al. (1974) have shown that nuclei from animals labelled for short periods of time (30-40 min) manifest this temperature- and energy-dependent release of RNA. Further, during early periods of incubation, the released RNA is essentially messenger-like, whereas the second phase of release is accounted for by rRNA. In the present study, a pre-labelling period of 4 h has been used and thus the time-sequence of release of the messenger-like and ribosomal-like RNA species cannot be expected. However, an attempt has been made to quantify the poly(A)-containing RNA in the released RNA fraction, and the results indicate that 2-allyl-2-isopropylacetamide treatment definitely enhances the release of poly(A)-containing RNA as well. However, the enhanced release of poly(A)-containing RNA does not entirely account for the increase in the release of total RNA. This might mean that the release of other RNA species, such as tRNA, rRNA etc., could also be enhanced in drug treatment under these conditions. However, the studies of labelling in vivo reveal that the immediate effect of 2-allyl-2-isopropylacetamide is to enhance labelling of poly(A)-containing RNA rather than that of rRNA. Further studies are needed to assess the effect of 2-allyl-2-isopropylacetamide treatment on the release of different RNA species. Polyacrylamide-gel-electrophoretic analysis of the poly(A)-containing RNA isolated from released RNA indicates that the bulk of the species shows a migration around 105 in normal animals, which agrees with the observations reported by Schumm & Webb (1974) and Shchuppe et al. (1974) on the basis of sucrose-density-gradient centrifugation. 2- Allyl-2-isopropylacetamide treatment appears to enhance the release of these poly(A)-containing RNA species as well as somewhat heavier species.

The gel-electrophoretic analysis of the cytoplasmic poly(A)-containing RNA labelled in vivo indicates that, under conditions of 2-allyl-2-isopropylacetamide

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treatment, there is a generalized increase in the labeling of a variety of species. The 2-allyl-2-isopropylacetamide-mediated enhanced labeling of nucleoplasmic RNA and cytoplasmic poly(A)-containing RNA species may be an indication of an increase in the amounts of messenger, not only for \( \delta \)-aminolaevulinate synthetase, but also for other proteins. Del Favero et al. (1975) have shown that 3,5-diethoxycarbonyl-1,4-dihydrocollidine causes a decrease in the proportion of single ("run off") ribosomes, and an increase in the number of polyribosomes, most probably owing to a relative increase in the amount of mRNA. A specific increase in the synthesis of one protein (such as \( \delta \)-aminolaevulinate synthetase) would result in a corresponding increase in amino acid incorporation by polyribosomes of the appropriate size. Since there is no evidence for any such peak of activity, it has been concluded that any changes are marked by increased synthesis of a number of other proteins (Del Favero et al., 1975).

A noteworthy feature of the results obtained with 2-allyl-2-isopropylacetamide is that haemin counteracts the drug-mediated increase in the \( ^{32} \)P-labelling of nucleoplasmic RNA (Sardana et al., 1975) as well as cytoplasmic poly(A)-containing RNA.

The financial assistance of the Council of Scientific and Industrial Research, New Delhi, is gratefully acknowledged.

References


