Br. J. Pharmac. (1970), **38**, 157–170.

Inhibition of the development of tolerance to morphine in rats by drugs which inhibit ribonucleic acid or protein synthesis

B. M. COX AND O. H. OSMAN*

Department of Pharmacology, Chelsea College, London S.W.3

Summary

1. A study has been made of the effects of a number of drugs which have as a common property the ability to inhibit RNA or protein synthesis, on the development of tolerance to the analgesic effects of morphine, given by intravenous infusion, in rats.

2. Measurements were also made of the effects of the drugs on the incorporation of ^{14}C-lysine into rat brain protein, and ^{14}C-orotic acid into rat brain RNA.

3. Actinomycin D, 6-mercaptopurine and 5-fluorouracil reduced the acquisition of tolerance to morphine at doses which also produced significant inhibition of incorporation of orotic acid into brain RNA. Tolerance development was only affected by 6-mercaptopurine and 5-fluorouracil after intracerebral administration.

4. 6-Mercaptopurine and 5-fluorouracil did not significantly affect the incorporation of lysine into protein, although a small but significant reduction in lysine incorporation was produced by infusion of actinomycin D at 20 μg/kg per hr.

5. Development of tolerance to the analgesic effects of morphine was also reduced by cycloheximide and puromycin. These drugs markedly reduced the incorporation of lysine into brain protein. Puromycin and low doses of cycloheximide (50 μg/kg per hr) did not significantly affect RNA synthesis as measured by orotic acid incorporation, but RNA synthesis was clearly reduced by administration of cycloheximide at 200 μg/kg per hr.

6. These results support the hypothesis that the synthesis of new RNA and protein in the brain is an essential feature of the development of tolerance to morphine in rats.

Introduction

It has recently been reported that the development of tolerance to some of the actions of morphine in rats or mice can be inhibited by such substances as actinomycin D (Cohen, Keats, Krivoy & Ungar, 1965 ; Cox, Ginsburg & Osman, 1968), 8-azaguanine (Spoerlin & Scrafani, 1967) and puromycin (Smith, Karmin & Gavitt,

* Present address: Department of Pharmacology, Faculty of Pharmacy, University of Khartoum, Khartoum, Sudan.

1966). A common property of these chemically diverse substances is that, directly or indirectly, they inhibit the biosynthesis of protein (see below). In an attempt to evaluate the hypothesis that inhibition of protein synthesis reduces or prevents the development of tolerance to morphine-like drugs, a study has been made of the effects on tolerance acquisition of a number of compounds which inhibit or modify protein synthesis. The effects of such drug treatments on the incorporation of precursors into brain RNA or protein have also been measured.

Drugs used in the present study (actinomycin D, 6-mercaptopurine, 5-fluorouracil, chloramphenicol, cycloheximide and puromycin) were selected to cover a wide range of mechanisms of protein synthesis inhibition. Actinomycin D binds to the guanine residues in DNA, thus preventing DNA directed RNA synthesis and hence *de novo* protein synthesis (Hamilton, Fuller & Reich, 1963 ; Goldberg & Reich, 1964). RNA synthesis is also modified by 6-mercaptopurine, which is thought to act principally by inhibiting the conversion of inosinate to adenylate and guanylate (Salser, Hutchison & Balis, 1960 ; Elion, 1967). 5-Fluorouracil is converted to 5-fluorodesoxyuridine monophosphate, which inhibits thymidine kinase and hence DNA synthesis (Heidelberger, 1965). There is also evidence that 5-fluorouracil is incorporated into RNA (Heidelberger, 1965), leading to the formation of proteins with modified structure and reduced biological activity (Naono & Gros, 1960 ; Bussard, Naono, Gros and Monod, 1960 ; Nemeth, 1962).

Chloramphenicol is reported to block peptide bond synthesis on bacterial ribosomes, and on 50 *S* subunits of bacterial ribosomes (Monro & Vasquez, 1967 ; Maden, Traut & Monro, 1968).

Cycloheximide probably also inhibits protein synthesis by an action on ribosomes preventing their movement along *m*RNA chains (Wettstein, Noll & Penman, 1964 ; Colombo, Felicetti & Baglioni, 1965), while puromycin appears to behave as an analogue of a *t*RNA-amino-acid complex which attaches to the growing polypeptide chain on the ribosome thus preventing further addition of amino-acids (Yarmolinsky & de la Haba, 1959). Nemeth & de la Haba (1962) consider that in mammalian liver puromycin also inhibits the formation of *t*RNA-amino-acid complexes.

A preliminary report of some of these results has been presented to the Biological Council Symposium on " The Scientific Basis of Drug Dependence " (Cox & Ginsburg, 1969) and to the British Pharmacological Society (Cox & Osman, 1969).

Methods

Preparation of animals

Male or female Wistar rats weighing between 140 and 200 g were prepared for intravenous infusion of solutions by the insertion of a polyethylene cannula into a jugular vein under ether anaesthesia (Cox *et al.*, 1968). The cannulae were inserted at least 18 hr before the rats were used.

Development of tolerance to the analgesic action of morphine

Drug solutions in 0·9% (w/v) NaCl were infused into unanaesthetized rats at a rate of 1 ml./hr. The duration of infusion was usually 7 hr. Before the infusion was started, and at various times during infusion, a measurement was made of threshold pressure at which the rat responded to a force exerted on the tip of its

tail by the plunger of a syringe connected to a compressed gas supply (Cox *et al.*, 1968). Each measurement was converted to an " index of analgesia " which was calculated by expressing the observed increase in pressure threshold as a proportion of the possible increase between the initial value before infusion and an arbitrary maximum pressure of 36 cm Hg.

Intracerebral injections of drugs

Rats were lightly anaesthetized with ether and unilateral injections were made through the skull at a point 2 mm lateral to the midline, and 2 mm anterior to an imaginary line joining the front of the ears. Examination of the skulls of injected animals indicates that this point corresponds closely to the point of injection used by Noble, Wurtman & Axelrod (1967). The needles (No. 20 gauge) were shortened to 4 mm in length, and the volume of injection was 50 μl. The rats recovered from the ether anaesthesia (to apparently normal locomotion) within 3 min.

In some rats injections of indian ink (diluted 1 : 10 with 0·9% NaCl) were made in this way. After 30 min the animals were killed, the brains removed, and fixed in formol saline. Examination of slices cut from the brains revealed that the injection needle penetrated the lateral ventricle. Some of the injected material entered the lateral ventricle, but ink also passed back up the needle track and spread rostrally and caudally through the subarachnoid space over parts of both the dorsal and ventral surfaces of the brain.

Incorporation of ^{14}C-lysine into brain protein

Two hours after the start of intravenous morphine or saline infusion, the rats were injected intracerebrally with 50 μl. of saline containing ^{14}C-lysine monohydrochloride (specific activity 2·5 μCi/ml. ; 11·7 μCi/mmol). Forty minutes later the animals were killed, the brain removed, blotted free of blood, and weighed. The brains were individually homogenized in 8 volumes of ice-cold 0·25 M sucrose solution using a Tri-R homogenizer with teflon pestle (clearance 0·006–0·009 inch). Samples of homogenate (5 ml.) were taken from each brain. To an aliquot (5 ml.) of homogenate was added 5 ml. 0·25 M sucrose and 1 ml. ice-cold 70% (w/v) perchloric acid (PCA), and the mixture was allowed to stand for 15 min in ice. It was then centrifuged at 12,000 *g* for 15 min at 0° C and the supernatant (S) set aside for the estimation of free lysine content and free radioactivity.

Protein was estimated gravimetrically after purification of the precipitate by the method of Shibko, Koivistoinen, Tratnyek, Newhall & Friedman (1967). This involved removal of ribonucleic acid by extraction with 0·3 N KOH at 37° C, extraction of DNA with 1% PCA at 90%, and removal of lipid with organic solvents. The protein pellet was finally dried from ether by heating at 105° C for 2 hr, then weighed exactly. The extracted protein was dissolved by heating in 1 N KOH (1 ml. KOH for 10 mg dry protein), in sealed test tubes to prevent evaporation. 1 ml. aliquots of the cooled solution were placed in stainless steel planchettes, dried under an infrared lamp, and the radioactivity was determined.

Determination of the specific activity of free lysine in the brain

The duplicate supernatants (S) from each animal were mixed together and passed through a Dowex 50W × 8 (H form) cation exchange resin to remove the perchloric acid, leaving the amino-acids bound to the resin. (A 20 × 1 cm column was used

for each brain supernatant, and a 6 cm length of wet resin was decanted into the column. Before use the resin was washed thoroughly with distilled water and then 5% PCA. The rate of flow was adjusted to about 0·5 ml./min.)

The amino-acids were eluted from the column with 25 ml. 4 N NH_4OH. Exactly 25 ml. of eluate was collected and two 1 ml. aliquots were placed in stainless steel planchettes and dried, and the radioactivity was determined. The remaining NH_4OH eluate was evaporated to dryness in a rotary evaporator under vacuum, the residue redissolved in 10 ml. distilled water, and the evaporation repeated to remove all traces of ammonia. The dry residue was dissolved in 2 ml. 0·01 N HCl, and the lysine content of 0·6 ml. samples determined by the lysine decarboxylase method of Hutzler, Odievre & Dancis (1967). Absorption at 400 mμ was measured with a Unicam S.P. 800 spectrophotometer.

Incorporation of ^{14}C-orotic acid into brain RNA

After a 2 hr intravenous morphine or saline infusion rats were injected intracerebrally with 50 μl. of saline containing 6-^{14}C-orotic acid (specific activity 2·5 μCi/ml ; 60·8 mCi/mmol). One hour later the animals were killed, the brains rapidly removed, blotted free of blood, weighed and homogenized individually in 5 volumes of 0·02 M sodium phosphate buffer pH 7·0 containing 0·1% sodium dodecylsulphate at 4° C. The RNA was extracted by the technique described by Barondes & Jarvik (1964) and estimated by measuring the absorption of the solution at 260 mμ on a Unicam S.P. 800 spectrophotometer. The orcinol method was found to give less consistent results. For determination of radioactivity 0·5 to 1 mg of RNA was precipitated on to a Millipore filter (pore size 0·45 mμ) with 2 volumes of 10% w/v trichloroacetic acid. The filter was placed in a planchette, dried and radioactivity measured.

Determination of the specific activity of uridine and uridine nucleotides

The brains from groups of six rats were weighed and homogenized in 7 volumes of distilled water at 4° C. Perchloric acid was added to a 20 ml. sample of the cold brain homogenate to give a final concentration of PCA of 6%. After centrifuging, the uridine and uridine nucleotides were extracted from the supernatant and estimated by the method described by Barondes & Jarvik (1964). Measurements of absorption at 262 mμ were made on a Unicam S.P. 800 spectrophotometer.

Measurement of radioactivity

Samples were dried, under an infrared lamp, in stainless steel or aluminium planchettes (depending on the sample solvent) and radioactivity determined in a Nuclear Chicago gas flow counter.

Drug and reagents

Actinomycin D (Dactinomycin) was generously donated by Merck, Sharp and Dohme, New Jersey. The following drugs were also used: 6-mercaptopurine hydrate (Sigma Chemical Co.), 5-fluorouracil (Koch-Light Laboratories Ltd.), chloramphenicol (Chloromycetin, Parke-Davis and Co.), cycloheximide (actidione, Nutritional Biochemicals Corp.), puromycin (Nutritional Biochemicals Corp.), morphine HCl. Lysine-^{14}C-monohydrochloride, and 6-^{14}C-orotic acid were obtained from the

Radiochemical Centre, Amersham. Lysine decarboxylase (Type I, crude acetone powder from *B. cadaveris*) and alkaline phosphatase (Type II from calf mucosa) were purchased from Sigma Chemical Co.

Except where otherwise indicated drugs were dissolved in aqueous 0·9% w/v NaCl. For intracerebral administration of 6-mercaptopurine it was necessary to heat the saline to 90° C to dissolve the drug. Injections were made with a warmed syringe when the solution had cooled to 37°–40° C. Crystallization did not occur until the solution had cooled to 20°–25° C.

Results

Inhibition of tolerance development during a single intravenous infusion of morphine

It has been shown previously that the intravenous infusion of morphine HCl to unanaesthetized rats at a rate of 7·5 mg/kg per hr produced a reduction in sensitivity to a pressure stimulus applied to the tail which was maximal 2–3 hr after the start of the morphine infusion. Thereafter sensitivity to the pressure stimulus gradually returned towards the normal level despite the continued administration of the drug. This decline in the analgesic effect of morphine was attributed to the

TABLE 1. *Effects of inhibitors of protein synthesis on the differences between analgesic indices a 2 and 7 hr after the start of infusion of a standard morphine solution*

Group	Treatment in addition to morphine	No. of animals	△2–7 values Means±S.E.	*P* value	Control group
1	Nil	10	0·80±0·01	—	—
2	Nil	8	0·78±0·02	>0·10	1
3	Nil	5	0·81±0·05	>0·10	1
4	Saline 50 μl. i.c. at 0 hr	6	0·74±0·04	>0·10	2
5	Saline 50 μl. i.c. at 0 hr	6	0·75±0·03	>0·10	2
6	Saline 3 i.c. injections of 50 μl. at −42 hr, −18 hr. and 0 hr	6	0·75±0·03	>0·10	2
7	Actinomycin D 10 μg/kg per hr i.v. throughout morphine infusion	8	0·14±0·07	<0·001	1
8	Actinomycin D 20 μg/kg per hr i.v. throughout morphine infusion	10	0·02±0·03	<0·001	1
9	Actinomycin D 1 μg/rat i.c. at 0 hr	8	0·08±0·04	<0·001	4
10	6-Mercaptopurine 2 mg/kg per hr. i.v. throughout morphine infusion	4	0·62±0·10	<0·10>0·05	2
11	6-Mercaptopurine two injections 5 mg/kg s.c. at −18 hr and 0 hr	4	0·71±0·05	>0·10	2
12	6-Mercaptopurine 200 μg/rat i.c. at 0 hr	6	0·28±0·07	<0·001	4
13	6-Mercaptopurine three injections of 100 μg/rat i.c. at −42, −18 and 0 hr	6	0·13±0·07	<0·001	6
14	5-Fluorouracil 5 mg/kg per hr i.v. throughout morphine infusion	5	0·72±0·03	>0·10	2
15	5-Fluorouracil 100 μg/rat i.c. at 0 hr	6	0·66±0·05	>0·10	4
16	5-Fluorouracil three i.c. injections of 200 μg/rat at −42, −18 and 0 hr	6	0·67±0·03	<0·10>0·05	6
17	5-Fluorouracil three i.c. injections of 400 μg/rat at −42, −18 and 0 hr	6	0·31±0·10	<0·002	6
18	Chloramphenicol 5 g/kg p.o. at −18 hr, +25 g/kg p.o. at 0 hr	4	0·81±0·02	>0·10	2
19	Chloramphenicol 4×300 mg/kg s.c. at 0, 2, 4, 6 hr	8	0·70±0·02	<0·05>0·02	2
20	Cycloheximide 50 μg/kg per hr. i.v. throughout morphine infusion	5	0·26±0·07	<0·001	3
21	Cycloheximide 200 μg/kg per hr i.v. throughout morphine infusion	5	0·00±0·00	<0·001	3
22	Puromycin 100 μg/rat i.c. at 0 hr	6	0·17±0·09	<0·001	5
23	Puromycin 200 μg/rat i.c. at 0 hr	6	0·16±0·05	<0·001	5

All rats received a standard infusion of morphine 7·5 mg/kg per hr intravenously for 7 hr. Times indicated in the treatment column refer to times from the start of the morphine infusion. Probability values have been calculated from Student's *t* test in comparison with the appropriate control group.

development of tolerance to the drug (Cox *et al.*, 1968). A measure of the degree of tolerance developed can be obtained by calculating for each animal the difference between the analgesic index values obtained at 2 and 7 hr after the start of the morphine infusion. We have called this difference the △ 2–7 value. Table 1 shows the effects of saline or drug treatments on the degree of tolerance (as estimated by the △ 2–7 value) developed during the standard morphine infusion of 7·5 mg/kg per hr. The mean △ 2–7 values calculated for three different groups of animals (Groups Nos. 1, 2 and 3) which received only the standard morphine infusion, are not significantly different. A single intracerebral injection of saline at the start of the morphine infusion (groups Nos. 4 and 5) or three intracerebral injections of saline before the morphine infusion (group No. 6) appeared to produce a very slight reduction in the degree of tolerance developed, but these effects were not statistically significant. The △ 2–7 values obtained from groups of animals receiving various drug treatments have been compared with the values obtained from appropriate control groups which received the drug vehicle (saline) on the same occasion.

The ability of actinomycin D (administered concurrently with the morphine infusion, at a rate of 10 μg/kg per hr) to reduce markedly the degree of tolerance developed has previously been reported (Cox *et al.*, 1968). An increase in the rate of infusion of the antibiotic to 20 μg/kg per hr completely prevented tolerance development (the △ 2–7 value was not significantly different from zero). A single intracerebral injection of 1 μg actinomycin D at the start of the morphine infusion was also almost completely effective in preventing the acquisition of tolerance (Table 1).

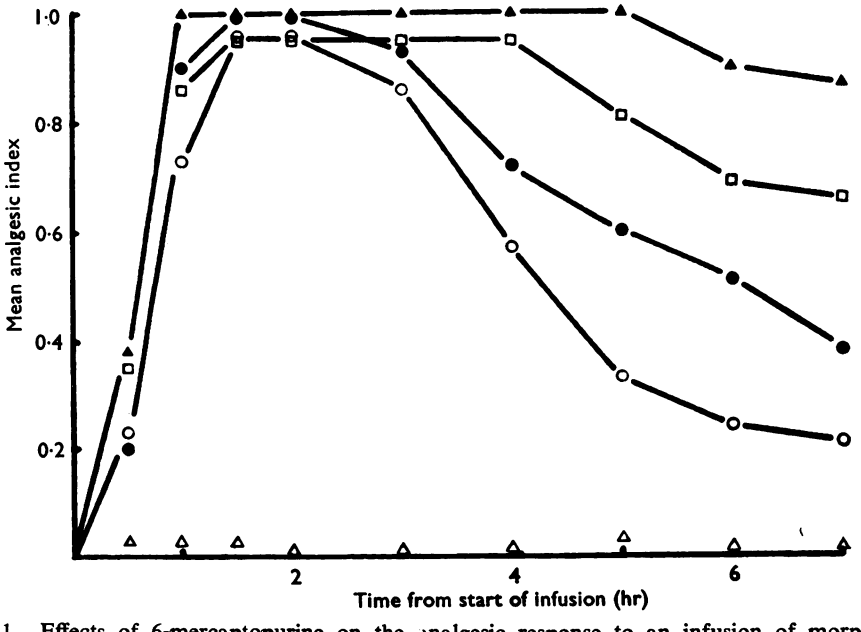

FIG. 1. Effects of 6-mercaptopurine on the analgesic response to an infusion of morphine. ⊙, Morphine 7·5 mg/kg per hr intravenously, plus saline intracerebally at start of the infusion ($n=6$); ●, 6-mercaptopurine 2 mg/kg per hr intravenously, plus morphine 7·5 mg/kg per hr ($n=4$); □, 6-mercaptopurine 200 μg intracerebrally at start of infusion of morphine 7·5 mg/kg per hr intravenously ($n=6$); ▲, three intracerebral injections of 6-mercaptopurine 100 μg at 42, 18 and 0 hr before infusion of morphine 7·5 mg/kg per hr intravenously ($n=6$); △, 6-mercaptopurine 200 μg intracerebrally at start of saline infusion 1 ml./hr ($n=5$).

Concurrent intravenous infusion of 6-mercaptopurine at a rate of 2 mg/kg per hr with morphine appeared to give a slight reduction in the decline of analgesic activity during the morphine infusion (Fig. 1), but this reduction was not significant ($P<0{\cdot}10>0{\cdot}05$). The low solubility of 6-mercaptopurine in aqueous solution prevented its administration intravenously at a higher rate. Subcutaneous injection of a suspension of the drug in 5% gum acacia at a dose of 5 mg/kg given 18 hr before the start of the morphine infusion, and a further 5 mg/kg subcutaneously at the start of the morphine infusion did not reduce the △ 2–7 value (Table 1). In view of the marked potency of actinomycin D after intracerebral administration, the effects of intracerebral injection of 6-mercaptopurine were investigated. It can be seen from Fig. 1 and Table 1 that 200 μg 6-mercaptopurine given intracerebrally at the start of the morphine infusion produced a 62% reduction in the degree of tolerance developed. An 83% reduction was produced by giving three intracerebral injections of 100 μg 6-mercaptopurine at 42 and 18 hr before, and at the start of the morphine infusion. 6-Mercaptopurine when given intravenously or intracerebrally in the absence of morphine showed no analgesic activity.

Tolerance development was not affected by intravenous infusion of 5-fluorouracil at a rate of 5 mg/kg per hr together with morphine. A single intracerebral injection of 100 μg at the start of the morphine infusion or three intracerebral injections of 200 μg before the morphine infusion were also ineffective. However, three intracerebral injections of 400 μg 5-fluorouracil reduced the △ 2–7 value by 59% ($P<0{\cdot}002$). This dose schedule did not exert analgesic activity in the absence of morphine.

The low aqueous solubility of chloramphenicol prevented its administration by the intravenous route. The oral administration of a suspension of the drug in 5% gum acacia at a dose of 5 g/kg 18 hr before the start of the morphine infusion and a further 2·5 g/kg at the start of the morphine infusion did not reduce the development of tolerance to the analgesic drug. Four subcutaneous injections of 300 μg/kg at the start of and at 2 hr intervals throughout the infusion period produced a very small reduction in the △ 2–7 value (Table 1).

Tolerance development was readily prevented by the simultaneous intravenous administration of cycloheximide with morphine. In an initial experiment a dose of 2 mg/kg per hr was used, but this was clearly toxic and the animals died between 4 and 8 hr after the start of the infusion. A △ 2–7 value of zero was recorded from animals which received cycloheximide at a rate of 200 μg/kg per hr together with morphine, and infusion at 50 μg/kg per hr produced a highly significant 68% inhibition in △ 2–7 value (Table 1). These doses were without analgesic activity and produced no gross behavioural signs of toxicity.

Intracerebral injections of 100 μg or 200 μg of puromycin to each rat at the start of the morphine infusion produced a very marked reduction in the degree of tolerance developed (Table 1). The effects of administration by other routes has not been tested because of the small quantity of drug available to us. In the absence of morphine puromycin treatment did not exert any analgesic effect.

Effects of inhibition of tolerance development on the response to a subsequent infusion of morphine

Previous experiments have shown that the administration of a 7 hr morphine infusion to rats leads to a considerable reduction in the maximum level of analgesia

attained during a second morphine infusion started 17 hr after the termination of the first (Cox *et al.*, 1968). When animals were given actinomycin D at a dose (20 μg/kg per hr) which prevented tolerance development during their first exposure to morphine, however, the maximum analgesic response recorded during a second infusion of morphine was almost as great as in rats which had not previously received morphine. In contrast, when a similar experiment was performed using cycloheximide (200 μg/kg per hr) to prevent acquisition of tolerance, the maximum level of analgesia developed during a second exposure to morphine was clearly lower than in animals previously given morphine and actinomycin D (Fig. 2). A χ^2 comparison was made of the total number of observations of failure to respond to the maximum pressure stimulus at 1·5, 2 and 3 hr after the start of the second morphine infusion, in animals from the two pretreatment groups. The number of responses to the pressure stimulus following the two pretreatments were significantly different ($P<0·05$).

Incorporation of ^{14}C-lysine into brain protein

Measurements were made of the incorporation of intracerebrally injected ^{14}C-lysine into the total protein of rat brain. The lysine was injected 2 hr after the start of an infusion of morphine or saline; the animals were killed and the brains removed and extracted 40 min later. These times were selected because it was between the second and third hour of a morphine infusion that tolerance first became detectable. The specific activity of free lysine in the brain was determined for two reasons; first, to enable compensation to be made for errors in the volume of labelled amino-

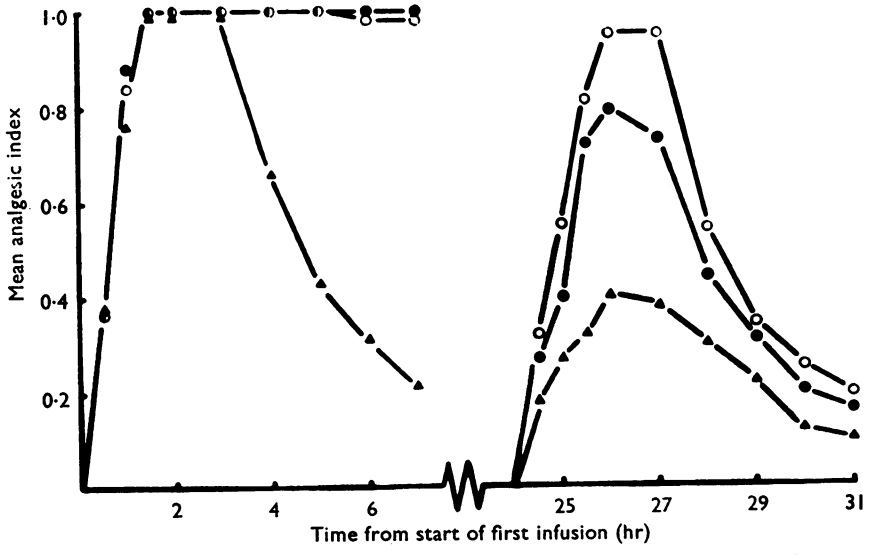

FIG. 2. Effects of inhibition of tolerance by actinomycin D or cycloheximide on the analgesic response to a subsequent infusion of morphine. The duration of both morphine infusions was 7 hr; the second infusion started 17 hr after the termination of the first. ▲, Morphine 7·5 mg/kg per hr intravenously only during both infusions ($n=11$); ○, actinomycin D 20 μg/kg per hr intravenously plus morphine 7·5 mg/kg per hr intravenously at the first infusion, morphine alone at the second infusion ($n=8$); ●, cycloheximide 200 μg/kg per hr intravenously plus morphine 7·5 mg/kg per hr intravenously at the first infusion, morphine alone at the second infusion ($n=5$).

acid solution injected, and, second, to allow compensation for variation in the incorporation of label into protein resulting from possible drug induced changes in the free amino-acid pool. Incorporation has therefore been expressed as a specific activity ratio which was calculated as the ratio of specific activity of brain protein (c.p.m./mg protein) to the specific activity of the free lysine in the brain (c.p.m./mg lysine).

Drug treatments were administered by the same route, and at the same time in relation to the morphine or saline infusion as in the previous experiments in which their effects on tolerance acquisition were investigated. Intravenous infusion of morphine produced an 11% reduction in the incorporation of lysine into total brain protein, but this effect was not statistically significant ($P>0.05$). Infusion of actinomycin D at a rate of 20 μg/kg per hr, with or without morphine, inhibited lysine incorporation by about 20%. However infusion of actinomycin D at a lower rate or administration of 6-mercaptopurine or 5-fluorouracil failed to produce significant inhibition of protein synthesis, although these treatments had resulted in a very clear inhibition of tolerance development (Table 2).

Lysine incorporation was inhibited by about 70% by intravenous infusion of cycloheximide at 200 μg/kg per hr. Infusion of the drug at 50 μg/kg per hr reduced incorporation by 18%, but this effect was not statistically significant. Intracerebral injection of 100 μg or 200 μg of puromycin reduced protein synthesis by about 40% and a slightly greater inhibition was obtained when morphine was infused concurrently (Table 2).

TABLE 2. *Effects of drugs on the incorporation of ^{14}C-lysine into rat brain protein*

Treatment	No. of observations	Mean c.p.m./mg protein	Mean c.p.m./mg free lysine $\times 10^{-5}$	Specific activity ratio (mean±S.E.) $\times 10^{4}$	% Inhibition	*P* value
Saline i.v. control	10	19·06	1·162	1·67±0·09	—	—
*Morphine 7·5 mg/kg per hr i.v.	7	17·68	1·221	1·49±0·10	11	<0·10>0·05
Actinomycin D 10 μg/kg per hr i.v.	6	18·17	1·200	1·53±0·09	9	>0·10
Actinomycin D 20 μg/kg per hr i.v.	7	16·74	1·314	1·29±0·06	23	<0·002
*Morphine 7·5+actinomycin D 20	6	17·60	1·347	1·33±0·12	20	<0·02
Saline i.c. and i.v. control	6	18·69	1·054	1·81±0·12	—	—
6-Mercaptopurine 200 μg i.c.	6	19·05	1·021	1·91±0·14	−5	>0·10
Saline three i.c. injections and i.v.	6	17·98	1·114	1·64±0·12	—	—
5-Fluorouracil 3×400 μg i.c.	6	16·28	1·081	1·56±0·14	2·5	>0·10
Saline i.v. control	5	18·85	1·204	1·61±0·20	—	—
Cycloheximide 50 μg/kg per hr i.v.	6	13·95	1·085	1·33±0·14	18	>0·10
Cycloheximide 200 μg/kg per hr i.v.	7	5·58	1·248	0·46±0·04	72	<0·001
*Morphine 7·5+cycloheximide 200	6	6·34	1·307	0·50±0·05	66	<0·001
Saline i.c. and i.v. control	6	19·27	1·138	1·70±0·12	—	—
Puromycin 100 μg i.c.	6	11·66	1·173	1·07±0·15	37	<0·01
Puromycin 200 μg i.c.	6	11·15	1·116	1·00±0·11	41	<0·001
*Morphine 7·5+puromycin 200 μg	6	9·40	1·129	0·87±0·08	49	<0·001

Specific activity ratio values were calculated individually for each animal. Probability values were determined from calculation of Student's *t*. All animals received an intravenous infusion of saline except those groups marked with an asterisk, which received an intravenous infusion of morphine.

Incorporation of ^{14}C-orotic acid into brain RNA

Orotic acid is converted to uridine monophosphate, which is then further phosphorylated to uridine diphosphate and triphosphate before its incorporation into RNA. It is thus possible that drugs may reduce the conversion of orotic acid to uridine monophosphate without affecting the rate of synthesis of RNA from purine and pyrimidine nucleotides. In using the incorporation of ^{14}C-orotic acid into RNA as a measure of RNA synthesis after drug treatment it is clearly necessary to show that the specific activity of the free uridine phosphates following administration of the labelled orotic acid is unchanged by the drug. Unfortunately methods available for the estimation of the free " uridine pool " (uridine derived from UMP, UDP and UTP, plus unphosphorylated uridine) were not sufficiently sensitive for this to be made in homogenates of single rat brains concurrently with measurements of incorporation into RNA. We have therefore measured the specific activity of the " uridine pool " in homogenates prepared from the brains of groups of six rats which had each received an intracerebral injection of ^{14}C-orotic acid (Table 3). None of the drug treatments have resulted in marked changes in the specific activity of the " uridine pool " ; it is probable that the small observed effects are all within the range of experimental error of the method.

TABLE 3. *Specific activity of the " uridine pool "*

Treatment	Specific activity c.p.m./mg uridine	% Change from control value
Saline i.v. only	3,600	—
Actinomycin D 20 μg/kg per hr i.v.	3,652	+1·5
Saline i.c.	3,973	—
6-Mercaptopurine 200 μg i.c.	4,148	+4·5
Saline three i.c. injections	4,724	—
5-Fluorouracil 3×400 μg i.c.	4,364	−7·5
Saline i.v. only	5,720	—
Cycloheximide 200 μg/kg per hr i.v.	5,650	−1
Saline i.c.	4,292	—
Puromycin 200 μg i.c.	4,533	+5·5

Uridine was estimated in the homogenate prepared from the brains of six animals. All animals received an intravenous infusion of saline (1 ml./hr). Two hours after the start of the saline infusion 0·125 μCi ^{14}C-orotic acid was injected intracerebrally. The animals were killed and brains removed one hour later.

TABLE 4. *Effect of drugs on incorporation of ^{14}C-orotic acid into total brain RNA*

Group No.	Treatment	No. of observations	Specific activity c.p.m./mg RNA (means±S.E.)	% Inhibition	*P* value
1	Saline i.v. control	8	1,031±54	—	—
2	Morphine 7·5 mg/kg per hr i.v.	6	1,034±65	0	>0·10
3	Actinomycin D 20 μg/kg per hr i.v.	6	863±54	17	<0·05
4	Actinomycin 1 μg i.c.	6	644±51	38	<0·001
5	Morphine 7·5+actinomycin D 20	6	801±48	22	<0·02
6	Saline i.c. and i.v. control	20	1,073±28	—	—
7	6-Mercaptopurine 200 μg i.c.	6	927±84	14	<0·05
8	5-Fluorouracil 3×400 μg i.c.	6	907±58	16	<0·05
9	Saline i.c. and i.v. control	15	1,379±49	—	—
10	Cycloheximide 50 μg/kg per hr	6	1,303±80	5	>0·10
11	Cycloheximide 200 μg/kg per hr	9	1,133±50	18	<0·01
12	Puromycin 200 μg i.c.	7	1,219±89	12	>0·10

All animals received an intravenous infusion of saline except groups Nos. 2 and 5, where this was replaced by an intravenous infusion of morphine 7·5 mg/kg per hr. Two hours after the start of the infusion 0·125 μCi ^{14}C-orotic acid was injected intracerebrally. The animals were killed and brains removed one hour later.

In the case of the results obtained from the animals treated with 5-fluorouracil it may be assumed the drug would have been converted to 5-fluorouridine phosphate (Heidelberger, 1965). We do not know whether the chromatographic technique used to isolate uridine in these experiments separates 5-fluorouridine from uridine, and it is therefore possible that in estimating the total uridine content of brains from 5-fluorouracil treated animals an error was introduced by the presence of 5-fluorouridine.

The drug treatments tested did not appear to influence significantly the conversion of orotic acid to uridine and uridine nucleotides, so the incorporation of labelled orotic acid into brain RNA has been used as an index of RNA synthesis (Table 4). The specific activity of brain RNA (c.p.m./mg RNA) was determined individually for each rat 1 hr after an intracerebral injection of orotic acid, which was given 2 hr after the start of a morphine or saline infusion. Drug treatments were administered by the same route, and at the same time in relation to the infusion as in the previous experiments.

RNA synthesis was unchanged by the intravenous infusion of morphine at 7·5 mg/kg per hr. A significant reduction of about 20% in orotic acid incorporation was produced by actinomycin D (20 μg/kg per hr intravenously) with or without a concurrent morphine infusion. A greater effect was produced by the intracerebral injection of 1 μg actinomycin D at the start of the saline infusion. Significant inhibition of RNA synthesis (by about 15%) was also produced by treatment with 6-mercaptopurine or 5-fluorouracil at doses which had been shown to reduce tolerance development. Intravenous infusion of cycloheximide at 50 μg/kg per hr, or the intracerebral injections of 200 μg puromycin, resulted in small but not statistically significant reductions in orotic acid incorporation. The intravenous infusion of cycloheximide at 200 μg/kg per hr, however, produced a statistically significant inhibition of RNA synthesis which was comparable in size to that produced by actinomycin D (20 μg/kg per hr, intravenously).

Discussion

The development of tolerance to the analgesic effects of morphine in rats was reduced or prevented by several drug treatments which also inhibited the synthesis of RNA or protein in the brain. It has previously been shown that actinomycin D does not affect the concentration of analgesic drug in the brain after intravenous infusion, and is more active by intracerebral than intravenous administration (Cox & Ginsburg, 1969). Two further observations support the conclusion that the metabolism of morphine was not reduced by drug treatments used in the present experiments. Both 6-mercaptopurine and 5-fluorouracil inhibited tolerance development after intracerebral but not after systemic administration. Second, rats which received a morphine infusion 17 hr after a previous infusion in which tolerance development was prevented with actinomycin D or cycloheximide showed a greater sensitivity to the morphine than rats which received morphine alone during the first infusion. If the inhibitors had been acting only by slowing the metabolism of morphine, then inhibitor-treated rats would be expected to be no more sensitive, and perhaps less sensitive, than the rats which previously received morphine alone.

The only drug tested which failed to produce a clear inhibition of tolerance development was chloramphenicol. This drug is an active inhibitor of protein synthesis in bacteria, and there is some evidence that it is a selective inhibitor of

mitochondrial protein synthesis in rat liver (Wheeldon & Lehninger, 1966). In mitochondrial or synaptosomal preparations from rat brain, however, chloramphenicol does not affect protein synthesis at concentrations up to five times greater than those required to inhibit liver mitochondrial protein synthesis (Gordon & Deanin, 1968). This may account for the failure of chloramphenicol to affect tolerance development.

The remaining drugs fall into two groups. At doses which produced marked reduction in tolerance development the ratio of protein synthesis inhibition to RNA synthesis inhibition was less than 1·5 for actinomycin D, 6-mercaptopurine and 5-fluorouracil (Table 5). In contrast, for cycloheximide and puromycin, the ratio was greater than 3. These observations thus support the view that actinomycin D, 6-mercaptopurine and 5-fluorouracil affect protein synthesis indirectly through inhibition of RNA synthesis while cycloheximide and puromycin act more directly on the assemblage of amino-acids into proteins. Both cycloheximide and puromycin treatment, however, appeared to produce some inhibition of RNA synthesis, although this was only statistically significant after the infusion of cycloheximide at a high rate. The inhibition of RNA synthesis by high doses of these drugs has previously been observed in mammalian tissues and is thought to be a secondary effect arising from the reduction in protein synthesis (Holland, 1963 ; Gorski & Axman, 1964).

All the drug treatments which inhibited tolerance development in the current experiments also showed a tendency to reduce the incorporation of orotic acid into brain RNA, although in some cases this effect was not statistically significant, so it is possible that prevention of tolerance acquisition is dependent solely on reduction of RNA synthesis.

Some inhibition of protein synthesis would be an inevitable sequel to this, but might not be related to drug action on the analgesic response. This possibility seems unlikely because the only biological role of RNA for which unequivocal evidence exists is in the direction of protein synthesis from information contained in DNA. (Volpe (1966) has argued that nucleic acids are concerned in synaptic transmission, but his evidence is not convincing.) Second, our observation that animals in which tolerance acquisition during a first exposure to morphine was prevented by either actinomycin D or cycloheximide showed differing sensitivities to morphine on a second exposure, implies a difference in the mechanism of action of the two inhibitor drugs. Thus animals pretreated with actinomycin D and morphine showed little evidence of tolerance to morphine when tested the next day, although some degree of tolerance was evident in animals pretreated with cycloheximide plus morphine. These results are consistent with the view that whilst actinomycin D was acting by inhibiting RNA synthesis, the principal action of cycloheximide was in reducing protein synthesis at the ribosomal level. Thus in

TABLE 5. *Relative inhibitory effect of drug treatments on RNA and protein synthesis*

Drug treatment	Ratio of % inhibition of lysine incorporation / % inhibition of orotic acid incorporation
Actinomycin D 20 μg/kg per hr i.v.	1·35
Actinomycin D 20 μg/kg per hr i.v. plus morphine 7·5 mg/kg per hr i.v.	0·91
6-Mercaptopurine 200 μg i.c.	0
5-Fluorouracil 3 × 400 μg i.c.	0·16
Cycloheximide 50 μg/kg per hr i.v.	3·5
Cycloheximide 200 μg/kg per hr i.v.	4·0
Puromycin 200 μg i.c.	3·4

the presence of cycloheximide RNA synthesized during exposure to morphine was unable to direct the formation of new protein. When the cycloheximide concentration fell to inactive levels, however, any of this RNA that had escaped metabolic degradation would be able to direct synthesis of protein(s) which might be responsible for the degree of tolerance observed in cycloheximide pretreated animals.

It would appear, therefore, that an essential component of the action of the drugs used in the present experiments to reduce tolerance development is inhibition of *de novo* protein synthesis. Measurements of the incorporation of ^{14}C-lysine into brain proteins over short periods of time (as used in the present experiments) are a poor indication of *de novo* protein synthesis because such incorporation will probably take place to a large extent on polyribosomes containing pre-existing messenger RNA. This may account for our failure to observe reduced lysine incorporation with indirectly acting inhibitors such as 6-mercaptopurine and 5-fluorouracil.

These arguments lead to the conclusion that the development of tolerance to the analgesic effects of morphine in rats is dependent on DNA directed RNA synthesis leading to the synthesis of new protein. This RNA synthesis appears to be particularly sensitive to inhibition by drugs, for tolerance development was prevented by inhibition of total RNA synthesis by only 15 to 20%. In the present experiments, however, morphine infusion did not result in significant changes in brain RNA or protein synthesis. We do not consider these results invalidate the hypothesis that an increase in the synthesis of a specific protein(s) is causally related to the development of tolerance, since such changes may be expected to occur in comparatively small regions of the brain in view of the selective nature of morphine action at low doses. Small localized increases in synthesis would not be detectable by measurement of overall incorporation into total brain RNA or protein. A similar criticism applies to the experiments of Clouet & Ratner (1967), who reported a reduction in the incorporation of ^{14}C-leucine into rat brain proteins after single intraperitoneal injections of morphine 60 mg/kg. Their observation of reduced incorporation may also result from a fall in neuronal activity which presumably accompanies the marked general depressant effects produced, in our experience, by morphine doses of this magnitude.

These experiments have confirmed that the development of tolerance to the analgesic effects of morphine in rats can be reduced or prevented by several drug treatments which, in different ways, inhibit the synthesis of RNA and/or protein in the brain. Tolerance development thus appears to require RNA and protein synthesis. However, the difficulty of interpreting experiments in which drug treatments result in severe disturbance of essential aspects of cell metabolism must be emphasized.

We should like to thank Professor M. Ginsburg for his advice and encouragement, and Dr. C. W. Mushett, of Merck, Sharp and Dohme Research Laboratories, New Jersey, U.S.A., for the generous supply of actinomycin D. The work reported here was undertaken by one of us (O. H. O.) in partial fulfilment of the requirements for the degree of Ph.D. in the University of London. O. H. O. was a British Council Scholar.

REFERENCES

BARONDES, S. H. & JARVIK, M. E. (1964). The influence of actinomycin D on brain RNA synthesis and on memory. *J. Neurochem.*, **11**, 187–195.

BUSSARD, A., NAONO, S., GROS, F. & MONOD, J. (1960). Effets d'un analogue de l'uracil sur les proprietes d'une proteine enzymatique synthetisée en sa présence. *C.r. hebd. Séanc. Acad. Sci., Paris*, **250**, 4049–4057.

CLOUET, D. H. & RATNER, M. (1967). The effect of the administration of morphine on protein synthesis in rat brain *in vivo*. *Pharmacologist*, **8**, 213.

COHEN, M., KEATS, A. S., KRIVOY, W. & UNGAR, G. (1965). Effect of actinomycin D on morphine tolerance. *Proc. Soc. exp. Biol. Med.*, **119**, 381–384.

COLOMBO, B., FELICETTI, L. & BAGLIONI, C. (1965). Inhibition of protein synthesis by cycloheximide in rabbit reticulocytes. *Biochem. biophys. res. Comm.*, **18**, 389–395.

COX, B. M. & GINSBURG, M. (1969). Is there a relationship between protein synthesis and tolerance to analgesic drugs? Biological Council Symposium *The Scientific Basis of Drug Dependence*. London: Churchill.

COX, B. M., GINSBURG, M. & OSMAN, O. H. (1968). Acute tolerance to narcotic analgesic drugs in rats. *Br. J. Pharmac. Chemother.*, **33**, 245–256.

COX, B. M. & OSMAN, O. H. (1969). The role of protein synthesis inhibition in the prevention of morphine tolerance. *Br. J. Pharmac.*, **35**, 373–374*P*.

ELION, G. B. (1967). Biochemistry and pharmacology of purine analogues. *Fedn Proc.*, **26**, 898–904.

GOLDBERG, I. H. & REICH, E. (1964). Actinomycin inhibition of RNA synthesis directed by DNA. *Fedn Proc.*, **23**, 958–964.

GORDON, M. W. & DEANIN, G. E. (1968). Protein synthesis by isolated rat brain mitochondria and synaptosomes. *J. biol. Chem.*, **243**, 4222–4226.

GORSKI, J. & AXMAN, M. C. (1964). Cycloheximide (actidione) inhibition of protein synthesis and the uterine response to oestrogen. *Arch. Biochem. Biophys.*, **105**, 517–520.

HAMILTON, L. D., FULLER, W. & REICH, E. (1963). X-ray diffraction and molecular model building studies of the interaction of actinomycin with nucleic acids. *Nature, Lond.*, **198**, 538–540.

HEIDELBERGER, C. (1965). Fluorinated pyrimidines. *Prog. Nucleic Acid Res. molec. Biol.*, **4**, 1–50.

HOLLAND, J. J. (1963). Effects of puromycin on RNA synthesis in mammalian cells. *Proc. natn Acad. Sci. U.S.*, **50**, 436–443.

HUTZLER, J., ODIEVRE, M. & DANCIS, J. (1967). Analysis for lysine, arginine, histidine and tyrosine in biological fluids. *Analyt. Biochem.*, **19**, 529–541.

MADEN, R. E. H., TRAUT, R. R. & MONRO, R. E. (1968). Ribosome catalysed peptidyl transfer: the polyphenylalanine system. *J. molec. Biol.*, **35**, 333–345.

MONRO, R. E. & VASQUEZ, D. (1967). Ribosome catalysed peptidyl transfer: effects of some inhibitors of protein synthesis. *J. molec. Biol.*, **28**, 161–165.

NAONO, S. & GROS, F. (1960). Synthèse par *E. coli*. d'une phosphatase modifiée en présence d'un analogue pyrimidique. *C.r. hebd. Séanc. Acad. Sci., Paris*, **250**, 3889–3891.

NEMETH, A. M. (1962). The effect of 5-fluorouracil on the developmental and adaptive formation of tryptophan pyrrolase. *J. biol. Chem.*, **237**, 3703–3706.

NEMETH, A. M. & DE LA HABA, G. (1962). The effect of puromycin on the developmental and adaptive formation of tryptophan pyrrolase. *J. biol. Chem.*, **237**, 1190–1193.

NOBLE, E. P., WURTMAN, R. J. & AXELROD, J. (1967). A simple and rapid method for injection of ^{3}H-norepinephrine into the lateral ventricle of the rat brain. *Life Sci., Oxford*, **6**, 281–291.

SALSER, J. S., HUTCHISON, D. J. & BALIS, M. E. (1960). Studies on the mechanism of action of 6-mercaptopurine in cell-free preparations. *J. biol. Chem.*, **235**, 429–432.

SHIBKO, S., KOIVISTOINEN, P., TRATNYEK, C. A., NEWHALL, A. R. & FRIEDMAN, L. (1967). A method for sequential quantitative separation and determination of protein, RNA, DNA, lipid, and glycogen from a single rat liver homogenate or from a subcellular fraction. *Analyt. Biochem.*, **19**, 514–528.

SMITH, A. A., KARMIN, M. & GAVITT, J. (1966). Blocking effect of puromycin, ethanol and chloroform on the development of tolerance to an opiate. *Biochem. Pharmac.*, **15**, 1877–1879.

SPOERLIN, M. T. & SCRAFANI, J. (1967). Effects of time and 8-azaguanine on the development of morphine tolerance. *Life Sci., Oxford*, **6**, 1549–1564.

VOLPE, P. (1966). About a possible participation of nucleic acids in synaptic transmission. *Experientia*, **22**, 615–617.

WETTSTEIN, F. O., NOLL, H. & PENMAN, S. (1964). Effect of cycloheximide on ribosomal aggregates engaged in protein synthesis *in vitro*. *Biochim. biophys. Acta*, **87**, 525–528.

WHEELDON, L. W. & LEHNINGER, A. L. (1966). Energy-linked synthesis and decay of membrane proteins in isolated rat liver mitochondria. *Biochemistry, N.Y.*, **5**, 3533–3545.

YARMOLINSKY, M. B. & DE LA HABA, G. (1959). Inhibition by puromycin of amino acid incorporation into protein. *Proc. natn Acad. Sci. U.S.*, **45**, 1721–1729.

(*Received July* 16, 1969)