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Effects of biochemical inhibitors on positional signalling in the chick limb bud

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SUMMARY

In 3- to 4-day embryonic chick limb buds, a region at the posterior margin of the limb, the zone of polarizing activity, appears to be responsible for signalling positional information along the anteroposterior axis. Our experiments were designed to test which biosynthetic processes are required for polarizing activity. We have treated polarizing regions with biochemical inhibitors, and then assayed their abilities to induce limb reduplications when grafted into anterior sites on host limb buds, and also measured their capacities for protein, RNA, and DNA synthesis. DNA synthesis, and possibly oxidative phosphorylation, do not seem to be required for polarizing activity. But, glycolysis and protein and RNA synthesis are necessary, although not sufficient, for polarizing region activity. Activity seems particularly sensitive to inhibitors (actinomycin D and α-amanitin) of RNA synthesis.

INTRODUCTION

There is a region of the posterior margin of the 3 to 4-day- embryonic chick limb bud which appears to be responsible for specifying positional information along the anteroposterior axis of the limb (Tickle, Summerbell & Wolpert, 1975). This region, the zone of polarizing activity, was originally identified by Saunders & Gasseling (1968). Following a graft of this polarizing region to the anterior margin of a 3- to 4-day embryo wing, the 10-day limb exhibits a mirror image reduplication, which is particularly striking in the hand, where instead of the normal pattern of three digits listed in anteroposterior sequence as **2 3 4** there may be present six digits in the pattern **4 3 2 2 3 4**. Tissues from the same region of other amniote species are capable of causing chick wing reduplication

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(MacCabe & Parker, 1976; Tickle, Shellswell, Crawley & Wolpert, 1976; Fallon & Crosby, 1977). The action of the polarizing region can be interpreted in terms of production of a graded signal, perhaps a diffusible morphogen, whose concentration falls off with distance (Tickle *et al.* 1975; Summerbell, 1979). If a grafted polarizing region has been 'weakened' by γ-irradiation (Smith, Tickle & Wolpert, 1978) or by dilution with non-polarizing region cells (Tickle & Goodman, personal communication), lesser reduplications such as **3 2 2 3 4** or **2 2 3 4** occur.

The nature of the signal from the polarizing region specifying new positional values is unknown, and it has, so far, been refractory to conventional analysis for lack of a suitable assay: no subcellular preparation has been reported to exhibit activity *in vivo*. MacCabe has developed an assay for the polarizing region based on its ability to maintain the apical ectodermal ridge *in vitro*, but it is not clear how this is related to positional signalling (Calandra & MacCabe, 1978).

In an effort to elucidate the molecular basis of the action of the polarizing region, we have treated isolated polarizing regions with a range of metabolic and biosynthetic inhibitors. These regions were grafted into the anterior margin of the chick wing bud and their ability to cause reduplications was examined. To help interpretation of these results we have assayed these small blocks of tissue for protein, RNA, and DNA synthesis and the ability of the cells to spread. When possible we have used irreversible inhibitors, because grafted polarizing regions can probably exert their effects 10–20 h after grafting although they must remain active for at least 10 h to specify additional digits (Smith, 1980). The experiments were designed to determine which biosynthetic processes are essential for the action of the polarizing region. We have tested inhibitors of metabolism and of protein, RNA and DNA synthesis. Ideally, we hoped to find that activity was either very sensitive to, or almost unaffected by, for example, decreased protein synthesis.

MATERIALS AND METHODS

Materials

Fertilized chicken eggs were obtained from the local flocks of Orchard Farm, Great Missenden, U.K., or Needle Farm, Enfield, U.K. Fertilized eggs of Japanese quail (*Coturnix coturnix japonica*) were obtained from a colony of the quail kept at the Middlesex Hospital Medical School.

All inhibitors were obtained from Sigma (London) except for potassium cyanide (Analar) and abrin and diphtheria toxin for which kind gifts we are grateful to Dr D. C. Edwards (Chester Beatty Research Institute, London).

Culture media were purchased from GIBCO (Paisley, U.K.) and were: Hank's Balanced Salts Solutions containing 20 mM HEPES, and 1 % Antibiotic-antimycotic solution (HBSS); and Minimal Essential Medium containing 10 %

Foetal Calf Serum, 20 mM L-glutamine, and 1 % antibiotic-antimycotic, with either Hank's Salts with 25 mM HEPES (HMEM) for use with an atmosphere of air, or Earle's Salts (EMEM) for use with an atmosphere of 5 % CO_2 in air.

Assay of polarizing region activity

Pieces of polarizing region tissue about 200 μm on a side were removed from stage 21–23 (Hamburger & Hamilton, 1951) chick limbs, placed in a dish of HBSS, pinned with a piece of 25 μm diameter platinum wire and treated, except when otherwise noted, for 1 h at 25 °C by immersion in 1 ml of HBSS containing inhibitor. They were then washed for 1 h with two changes of 1 ml HBSS each, and grafted into host chick wing buds at stage 19 or 20 opposite somite 16. Five to ten polarizing region grafts were done for each experimental point. Operated embryos were fixed, stained (Summerbell & Wolpert, 1973) and examined after six days further incubation at 38 °C. In control operations polarizing regions were manipulated through sham treatment and wash steps before grafting; these gave full **4 3 2 2 3 4** or **4 3 2 3 4** reduplications (Fig. 1*b*) in about 90 % of the cases.

Polarizing region activity was quantified through a weighted average. Each operated embryo in a set was assigned 0, 1, 2 or 3 points depending on whether it had no reduplicated digits (0 points), or a reduplication with an induced extra digit **4** (3 points), a **3** (2 points), or only a **2** (1 point) as shown in Fig. 1. The totalled number of points achieved by a set of embryos was divided by the total numbers of points possible (3 points for each embryo, i.e. all **4** reduplications), to yield, a percentage which was 100 % if all the operated limbs exhibited reduplications containing induced digits **4**, and 0 % if no limbs were reduplicated. This scale of polarizing activity can only be linear if the thresholds for specification of digits **2**, **3**, and **4**, are equally spaced. Control sets of embryos, into which sham-treated polarizing regions were grafted, gave activities of 85–100 %.

Assay of cell spreading

Excised polarizing regions were treated and washed as described above, except that they were not pinned and grafted, but cut into four pieces and placed in 35 mm plastic tissue culture dishes (Sterilin) containing 1 ml HMEM, and examined after 24 h incubation at 38 °C. Under these conditions, normal polarizing regions attached to the surface of the dish and flattened cells spread out from the periphery of the pieces of tissue. For each experimental set spreading after 24 h was qualitatively scored as 0 if no flattened spread cells were apparent, or as 1 to 3 plusses (+) reflecting the number and flatness of the spread cells. Control polarizing regions, assayed each experiment, always scored + + +.

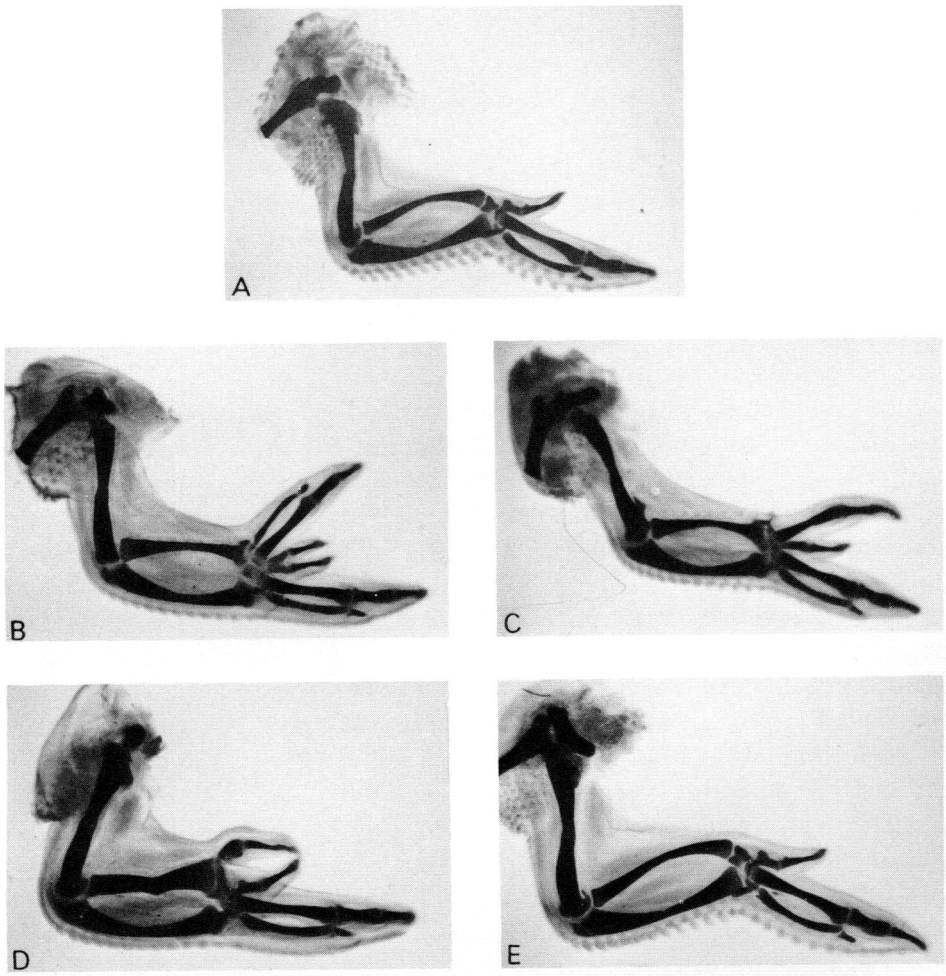

Fig. 1. Whole mounts of 10-day embryonic chick right wings (magnifications approx. ×6). (*a*) Normal, unoperated wing with digit pattern **2 3 4**. (*b*) Fully reduplicated (**4 3 2 2 3 4**) wing following graft of a sham-treated polarizing region. (*c*) Wing with digit pattern **3 2 3 4**, following graft of a polarizing region treated with 1·5 ng/ml abrin for 3 h at 38 °C in EMEM. (*d*) Wing with digit pattern **2 2 3 4** following graft of a polarizing region treated with 1 mM CCCP for 1 h at 25 °C in HBSS. (*e*) Wing with normal digit pattern (**2 3 4**) following graft of a polarizing region treated with 1 μg/ml actinomycin D for 1 h at 25 °C in HBSS.

Biochemical assays

For each experiment, several hundred pieces of polarizing region were excised in HBSS from stage-21 to -24 embryo wing and leg buds (one or two pieces from the polarizing region were taken from each limb). The polarizing regions were distributed equally by stage into the wells (15–20 per well) of a microtiter plate (Flow Lab. No. 76-201-05), incubated in inhibitor as above, using two wells for each experimental point, and washed for 1 h with 4 changes of 150 μl HBSS or EMEM. At this time, or after further incubation at 38 °C in inhibitor-

free medium, were added 50 μl of EMEM containing (*a*) L-[4,5-^{3}H]leucine to label protein, (*b*) [5,6-^{3}H]uridine, with 20 μM each 2′-deoxycytidine and thymidine to label only RNA, or (*c*) [6-^{3}H]thymidine to label DNA. The radioisotopes were obtained from Amersham at specific activities of 51, 43, and 39 Ci/m-mole respectively and used at 50–100 μCi/ml. The plates were incubated at 38 °C for 2 to 4 h over which time synthesis in controls was linear. Synthesis was stopped by transfer on to ice, and the tissue pieces were washed for 1 h with four rinses (150 μl each) of charcoal-decolorized HBSS without HEPES, containing an excess (1 mM) of cold precursor. The polarizing regions were then frozen, thawed, and disrupted by 1 min sonication on ice with the Dawe Soniprobe Type 1130A at position 2. Aliquots of total suspension were placed on Whatman GF/C filters, and equal aliquots of material were, together with yeast RNA carrier, acid-precipitated by the addition of trichloroacetic acid to 7·5 % (w/v), filtered, and washed with 5 % trichloroacetic acid through GF/C filters. All filters were air-dried at 60 °C for about 8 h, and liquid scintillation counted using a scintillation fluid consisting of 0·5 % PPO and 0·01 % POPOP in toluene. Uptake of precursors in acid-soluble form was taken as the difference between total and acid-precipitated counts.

Duplicate aliquots of suspension were also taken for protein assays by the Coomasie Blue-binding assay of Sedmak & Grossberg (1977), using a sample volume of 224 μl. The protein assay was sensitive to 0·2 μg protein and linear to 10 μg; duplicate points differed on average by 10 %. Variation between two duplicate wells was somewhat greater, but the final pooled standard deviation for any experimental point based on the two duplicate wells, each with duplicate protein determination, was usually less than 10 %. The data for synthesis and uptake, initially expressed as p-mole precursor per mg tissue protein per hour are here presented as percentage of the control, sham-treated, figures of each experiment. For all three precursors, the actual control values were usually about 100–200 p-mole/mg/h incorporated into acid-precipitable material.

Rates of incorporation of radio-labelled amino acids or nucleotides precursors into acid-precipitable material are frequently taken as indices, by themselves, of protein or nucleic acid synthesis, but such assumptions only hold when it is shown that precursor pool specific activities are the same in the cases compared. Especially with some treatments, inhibition of transport, and thus lowered pool specific activity, will spuriously appear to be a decrease in macromolecular synthesis. Although we did not measure precursor pools in our experiments, the uptake figures, representing the difference between acid-precipitated and total tissue counts may be used as a guide to interpretation of the results. If the uptake of acid-soluble counts was inhibited by an equal or greater extent than that of acid-precipitable counts, then it was possible that not synthesis, but maybe simply transport, was inhibited. On the other hand, if acid-precipitable incorporation was affected to a much greater extent than uptake of acid-soluble counts, then the simplest interpretation was that

biosynthesis, and not transport, was the primary target of the inhibitor. In these studies, inhibition of protein synthesis seemed to be invariably accompanied by at least some diminution of leucine uptake, while nucleoside uptake was not much reduced by inhibition of RNA synthesis alone.

RESULTS

Metabolic inhibitors

We treated polarizing regions for 1 h at 25 °C with potassium cyanide, which reversibly inhibits oxidative phosphorylation through its binding to cytochrome oxidase, carbonyl cyanide m-chlorophenylhydrazone (CCCP), which irreversibly uncouples oxidative phosphorylation, and iodoacetamide, an alkylating agent which irreversibly inactivates phosphofructokinase, a key glycolytic enzyme. The results can be seen in Table 1. Potassium cyanide had only slight effects on polarizing region activity and spreading, and on protein, RNA, and DNA synthesis, at concentrations which would be expected to inhibit oxidative phosphorylation (Slater, 1967). However, this result does not necessarily indicate that oxidative metabolism is not required for polarizing activity since the effect of cyanide is reversible. CCCP had little effect on polarizing region activity over its expected effective range of 1–10 μM (Slater, 1967). At higher concentrations (1 mM) CCCP considerably reduced activity, abolished spreading and inhibited DNA, RNA, and especially protein synthesis. When the incorporation data is viewed together with the uptake figures, it appears that the reduced nucleoside incorporation may have been primarily due to inhibition of transport but that protein synthesis was perhaps directly inhibited. That polarizing activity was not completely abolished even at high doses of CCCP having such severe 'non-specific' effects suggests that oxidative metabolism may not be required for polarizing region activity.

Iodoacetamide completely abolished polarizing region activity as well as spreading, protein, RNA, and DNA synthesis. The effect on polarizing region activity was apparent at lower concentrations (100 μM) than the major effects on macromolecular synthesis and spreading. It seems plausible that the effect on glycolysis is responsible for the inhibition of polarizing region activity. Iodoacetic acid had similar effects on polarizing region activity to those of iodoacetamide except that at 0·1 mM and even at 1 mM, 20 % activity persisted (data not shown).

Inhibition of protein synthesis

Polarizing regions were treated with the following inhibitors of protein synthesis: puromycin, which competes with incoming tRNA molecules and blocks the growing ends of nascent polypeptide chains (Vázquez, 1979); emetine, which binds to ribosomes inhibiting translocation (Vásquez, 1979); abrin, which catalytically modifies ribosomes preventing translocation (Olsnes & Pihl, 1977);

Table 1. *The effects of inhibitors of metabolism*

Potassium cyanide						
Concentration (mM)		0·1	1·0	10·0		
Polarizing region activity (%)		80	78	67		
Spreading (%)		+ +	+ +	+ +		
Protein synthesis (%)		—	71	82		
[^{3}H]leucine uptake (%)		—	87	83		
RNA synthesis (%)		—	151	113		
[^{3}H]uridine uptake (%)		—	118	95		
DNA synthesis (%)		—	123	130		
[^{3}H]thymidine uptake (%)		—	128	117		
Carbonyl cyanide m-chlorophenylhydrazone (CCCP)						
Concentration (μM)	1	5	10	100	500	1000
Polarizing region activity (%)	83	100	100	63	53	28
Spreading	+ + +	+ + +	+ + +	+ + +	+	0
Protein synthesis (%)	—	—	—	31	—	2
[^{3}H]leucine uptake (%)	—	—	—	36	—	7
RNA synthesis (%)	—	—	—	77	—	16
[^{3}H]uridine uptake (%)	—	—	—	63	—	9
DNA synthesis (%)	—	—	—	59	—	39
[^{3}H]thymidine uptake (%)	—	—	—	35	—	20
Iodoacetamide						
Concentration (μM)	10	30	100	300	1000	
Polarizing region activity (%)	61	61	6	0	0	
Spreading	+ + +	+ +	+	+	0	
Protein synthesis (%)	93	—	72	—	1	
[^{3}H]leucine uptake (%)	—	—	106	—	23	
RNA synthesis (%)	149	—	79	—	2	
[^{3}H]uridine uptake (%)	79	—	118	—	12	
DNA synthesis (%)	98	73	33	8	2	
[^{3}H]thymidine uptake (%)	151	124	82	32	12	

Polarizing regions were treated, for 1 h at 25 °C, as described in the text, in solutions of inhibitor in HBSS. For CCCP, the 10 mM stock solution, from which dilutions were made, was in ethanol. In a control experiment, treatment with 10% ethanol resulted in 75% polarizing activity and + + + spreading. Periods of incubation with label were 3 h for KCN and CCCP and 3·5 h for iodoacetamide experiments.

and diptheria toxin which, in susceptible cells, stops chain elongation through catalytic inactivation of Elongation Factor 2 (Collier, 1977).

The results are shown in Table 2. At concentrations which reduce protein synthesis, all these inhibitors attenuated polarizing activity. However, upon close comparison of the inhibitions of protein synthesis and polarizing activity, activity seemed less sensitive to puromycin, more sensitive to emetine, and most sensitive to abrin.

To investigate these differences, we examined the time course of synthesis

Table 2. *The effects of inhibitors of protein synthesis*

Puromycin					
Concentration (mg/ml)	0·06	0·25	1·0	4·0	
Polarizing region activity (%)	89	50	38	22	
Spreading	+++	+++	++	0	
Protein synthesis (%)	68	7	4	1	
[^{3}H]leucine uptake (%)	36	11	17	11	
RNA synthesis (%)	—	92	—	77	
[^{3}H]uridine uptake (%)	—	94	—	141	
DNA synthesis (%)	—	47	—	18	
[^{3}H]thymidine uptake (%)	—	54	—	66	
Abrin					
Concentration (mg/ml)	0·15	1·5	15	150	1500
Polarizing region activity (%)	96	67	0	—	—
Spreading	+++	+++	+++	+++	++
Protein synthesis (%)	—	70	36	13	3
[^{3}H]leucine uptake (%)	—	68	46	21	15
DNA synthesis (%)	—	98	70	34	—
[^{3}H]thymidine uptake (%)	—	92	99	60	—
Emetine					
Concentration (μM)	0·1	1·0	10	100	1000
Polarizing region activity (%)	—	100	22	0	0
Spreading	++	+	0	0	0
Protein synthesis (%)	116	57	12	5	5
[^{3}H]leucine uptake (%)	96	81	29	24	22
RNA synthesis (%)	—	110	—	—	81
[^{3}H]uridine uptake (%)	—	131	—	—	143
DNA synthesis (%)	—	68	—	—	16
[^{3}H]thymidine uptake (%)	—	120	—	—	83
Diptheria Toxin					
Concentration (ng/ml)	0·015	1·5	150	1500	15000
Polarizing region activity (%)	89	73	56	83	50
Spreading	++	++	++	++	+
Protein synthesis (%)	120	60	62	—	24
[^{3}H]leucine uptake (%)	101	44	27	—	16
RNA synthesis (%)	108	100	117	—	125
[^{3}H]uridine uptake (%)	117	98	125	—	133

Treatments were as described in the text except for abrin, with which treatment was for 3 h at 38 °C in EMEM. Sham treatment of polarizing regions for 3 h at 38 °C did not affect activity or spreading. Treatment temperature did not seem that important for diptheria toxin, and even after treatment with 15 μg/ml for 5 h at 38 °C, protein synthesis was 9% of control levels.

following treatment with inhibitors. Protein synthesis in polarizing tissue treated with 1 mg/ml puromycin significantly recovered such that while protein synthesis was initially 5% of control, it recovered to 29% after 5 h, and 47% after 13 h. Contrariwise, treatment with abrin, followed by wash with medium

Table 3. *The effects of inhibitors of RNA synthesis*

Actinomycin D Concentration (μg/ml)	0·01	0·05	0·1	0·3	0·5	1·0	2·0	5·0	10	20
Polarizing region activity (%)	67	88	33	35	27	0	—	0	6	13
Spreading	+++	+++	++	++	+	0	0	0	0	0
Protein synthesis (%)	—	116	—	—	—	105	106	—	—	—
[^{3}H]leucine uptake (%)	—	41	—	—	—	90	46	—	—	—
RNA synthesis (%)	92	97	108	73	84	75	35	18	10	—
[^{3}H]uridine uptake (%)	85	81	90	101	123	115	146	121	163	—
DNA synthesis (%)	—	109	—	—	—	107	123	—	65	—
[^{3}H]thymidine uptake (%)	—	126	—	—	—	114	137	—	85	—

α-Amanitin Concentration (μg/ml)	0·005	0·05	0·5	5·0	50
Polarizing region activity (%)	45	38	39	38	0
Spreading	+++	+++	+++	+++	0
Protein synthesis (%)	—	—	110	—	89
[^{3}H]leucine uptake (%)	—	—	106	—	91
RNA synthesis (%)	110	129	99	89	73
[^{3}H]uridine uptake (%)	76	73	78	82	90
DNA synthesis (%)	—	—	79	—	81
[^{3}H]thymidine uptake (%)	—	—	113	—	70

Treatments with actinomycin D were for 1 h at 25 °C in HBSS; the table represents the pooled data of several experiments. Treatments with α-amanitin were for 2 h at 38 °C in EMEM. Control treatment of polarizing regions for 2 h at 38 °C in EMEM had no effect on activity or spreading. Periods of incubation with label were 2·5 h.

resulted in continued decline of protein synthesis rather than recovery (which observation is consistent with the data of Eiklid, Olsnes & Pihl, 1980, for HeLa cells). In one experiment polarizing regions were treated for only 2 h at 38 °C with 1·5 ng/ml abrin, and washed for 1 hr after which protein synthesis was 109 ± 21 % of the control sham-treated tissue; but 5 h later the treated tissue synthesized only 47 ± 14 % of controls; after 15 h synthesis was at 61 ± 4 % of control tissue level. Although we have not measured recovery from emetine, the effects of this non-catalytic agent are usually rapid, and relatively irreversible (Grollman, 1968).

Bearing in mind that the effects of puromycin were partially reversible while those of abrin and emetine were relatively irreversible, as described above, the data in Table 2 suggests that inhibition of protein synthesis causes reduction of polarizing region activity. These agents inhibited RNA synthesis very little although DNA synthesis was reduced. This latter effect is frequently observed

as a secondary effect of inhibition of protein synthesis (Grollman, 1968; Lin *et al.* 1970; Brooks, 1977) but the results presented below suggest that inhibition of DNA synthesis does not, in itself, attenuate polarizing activity.

The 3-day embryonic chick cells were very resistant to diptheria toxin. This has not previously been reported but may indicate that the cells have very few receptors for the toxin B-chain, since all cells tested from 11- and 15-day chick embryos are very sensitive to diptheria toxin (Solotorovsky & Gabliks, 1965).

Inhibitors of RNA synthesis

Two inhibitors of RNA synthesis were used: actinomycin D, which binds to DNA preventing transcription, and at low concentrations selectively inhibits rRNA synthesis (Perry & Kelley, 1970), and α-amanitin which binds specifically to eucaryotic RNA polymerase II, and thus at low concentrations selectively inhibits mRNA synthesis (Lindell *et al.* 1970). The results are presented in Table 3. Actinomycin D considerably reduced polarizing activity at concentrations of 0·1 μg/ml and above, but only concentrations greater than 1·0 μg/ml significantly inhibited RNA synthesis. Similarly, α-amanitin partially inhibited polarizing activity at a concentration as low as 0·005 μg/ml, at which concentration bulk RNA synthesis was not reduced. Even after treatment with 50 μg/ml α-amanitin, which completely abolished polarizing activity, RNA synthesis was not diminished by more than 30 %. Neither uptake of precursors, nor synthesis of DNA or protein were greatly inhibited at doses of the drugs at which polarizing activity was eliminated. The results suggest either that some small sensitive RNA subclass is important for polarizing activity or that some other common non-specific effect is, at low concentrations of these drugs, inhibiting polarizing activity.

Actinomycin D appears to have less powerful effects on RNA synthesis in polarizing region cells than on other cells in culture (Sawicki & Godman, 1971) or on cultured early mouse embryos (Monesi, Molinaro, Spalletta & Davoli, 1970). Several experiments were performed to examine whether either its speed of action, or recovery from the drug might be responsible for this observation. In one experiment, three procedures were compared: (*a*) conventional 60-min treatment at 25 °C followed by wash, and a 150 min labelling period in the absence of drug: (*b*) presence throughout labelling period as well; and (*c*) sham initial treatment, with drug present only during the labelling period. The RNA synthesis profiles as a function of drug concentration were similar for all three treatments although the presence of actinomycin D throughout (*b* above) caused slightly more inhibition than the other treatments. In another experiment the effects of actinomycin D on RNA synthesis were found to be slightly reversible over 5–15 h. Protein synthesis was not significantly affected until 15 h following 1 h treatment with 1 μg/ml of the drug and then only 25 % reduced.

Table 4. *The effects of γ-irradiation*

Dose (rad)	10000	24000	64000
Polarizing region activity (%)	89	60	31
Spreading	+++	+++	0
Protein synthesis (%)	73	78	41
[^{3}H]leucine uptake (%)	128	117	80
RNA synthesis (%)	91	105	29
[^{3}H]uridine uptake (%)	116	111	42
DNA synthesis (%)	37	29	9
[^{3}H]thymidine uptake (%)	41	33	13

In this experiment, quail embryos were irradiated *in ovo* using a Vickers-Armstrong Mark IV ^{60}Co irradiation source at a dose rate of 1500 rad/min. Embryos were removed from their eggs into HBSS within 30 min following irradiation. Polarizing regions were removed and assayed by the same procedure that normally followed treatment and wash, for chemical inhibitors. Period of incubation with label was 4·2 h. Polarizing activity and spreading data are from experiments in this laboratory (Smith *et al.* 1978; Smith, 1979) using quail eggs from the same flock and irradiated in the same manner. A dose of 2500 rad causes 100% lethality of embryos following 2 days further incubation.

γ-Irradiation

High doses of γ-irradiation to quail polarizing regions have been shown to attenuate activity (Smith *et al.* 1978). Table 4 shows the levels of quail polarizing activity, protein, RNA and DNA synthesis after increasing doses of ^{60}Co γ-irradiation. Activity was not inhibited by 10000 rad irradiation while DNA synthesis was severely reduced.

DISCUSSION

The aim of this investigation was to examine the effects of various biochemical inhibitors on polarizing region activity, and to attempt to correlate this with some basic biosynthetic processes. But, there are a number of factors which make interpretation of our results difficult. Firstly, there is the possibility that an inhibitor may have toxic side-effects on processes that we have not examined (but see below). Secondly, as we have pointed out, the polarizing region takes time of the order of 15 h to act, and the effects of some of our inhibitors are reversible. Thirdly, we cannot be sure that despite our washing procedure, some inhibitor does not leak and affect responding tissue. (Although for the particular inhibitors used in this study, polarizing activity assays using disaggregated cells make this possibility seem unlikely; Honig, in preparation). Finally, when polarizing region activity is inhibited, we do not know if the effect is on the production of the signal or on its transmission. For example, an inhibitor could either inhibit synthesis of a morphogen, or the formation of gap junctions which might provide the channel for communication.

A general feature of the results is that polarizing activity is reduced with increasing concentrations of inhibitions. The changed pattern of extra digits following treatment of the grafted polarizing region with inhibitor is always of the type where the loss involves digit **4**, or digits **4 3** or all three extra digits, **4 3 2**. Nevertheless, there were relatively few limbs with patterns **3 2 2 3 4** or **2 2 3 4** compared with the number of **4 3 2 2 3 4** or **2 3 4**. For example, when polarizing activity was reduced to between 33 % and 66 %, then the percentage of limbs whose additional anterior digit was either none, **2**, **3** or **4**, was 41 %, 13 %, 11 %, and 35 % respectively. This reflects the observation that following certain treatments the observed patterns consisted of mostly either limbs containing no extra digits or complete reduplications.

Since reductions in number of grafted polarizing region cells results in decreased activity (Tickle & Goodman, personal communication), a possible explanation for the effects of inhibitors is that all they are doing is killing cells of the polarizing region. One index for cell viability is the spreading assay. Mostly, the inhibition of polarizing activity went hand-in-hand with inhibition of spreading. However, there were some exceptions: abrin abolished polarizing activity at a concentration which had little effect on spreading, while emetine had less effect on polarizing activity than on spreading. Also, when chick and quail polarizing regions differed in their sensitivity to inhibitors, their polarizing activity and spreading often did not differ in the same direction (unpublished results, and Smith, 1979). Another index of viability is macromolecular synthesis – it can be seen that in some cases polarizing activity was severely inhibited while one or more biosynthetic process was relatively unaffected as, for example, treatment with actinomycin D or α-amanitin. This also suggests that the effect is not *via* cell death.

Usually, inhibition of polarizing activity went in parallel with the inhibition of protein, RNA, and DNA synthesis, but there were two types of situation which could be informative. In one, synthesis would appear unaffected yet polarizing activity severely reduced: this indicates that the synthesis is not sufficient for polarizing activity. The other case is when polarizing activity would appear relatively unaffected yet synthesis would be at very low levels. This case is more interesting since it implies that the synthesis is not required for polarizing activity.

On this basis, DNA synthesis does not seem to be required for positional signalling, because at doses of γ-irradiation which did not significantly affect polarizing activity, DNA synthesis was reduced by over 60 %. It also appears that DNA synthesis can be at normal levels when polarizing activity is abolished, for example with actinomycin D and α-amanitin. Protein synthesis appears to be necessary for polarizing activity because its inhibition always led to coordinate inhibition of polarizing activity. But, once again, a normal level of protein synthesis is not a sufficient condition, since with actinomycin D and α-amanitin, protein synthesis was near untreated control values when polarizing

activity was abolished. For RNA synthesis, the situation is peculiar since specific inhibitors of RNA synthesis inhibited polarizing activity at concentrations where bulk RNA synthesis was relatively unaffected. This is true for both actinomycin D and α-amanitin, whose presumed primary targets are different – ribosomal RNA and messenger RNA respectively. Moreover these drugs had little effect on protein and DNA synthesis. This suggests that either some small subclass of RNA is sensitive to both inhibitors, and essential for polarizing activity, or that they both exert some unknown non-specific effect. It is possible that an indirect effect on a small subclass of RNA might also account for the effects of iodoacetamide: polarizing activity was severely inhibited but protein and RNA synthesis were not.

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REFERENCES

BROOKS, R. F. (1977). Continuous protein synthesis is required to maintain the probability of entry into S phase. *Cell* **12**, 311–317.

CALANDRA, A. J. & MACCABE, J. A. (1978). The *in vitro* maintenance of the limb bud apical ridge by cell-free preparations. *Devl Biol.* **62**, 258–269.

COLLIER, R. J. (1977). Inhibition of protein synthesis by exotoxins from *Corynebacterium diptheriae* and *Pseudomonas aeruginosa*. In *The Specificity and Action of Animal, Bacterial and Plant Toxins* (ed. P. Cuatrecasas), pp. 67–98. *Receptors and Recognition*, Series B, vol. 1. London: Chapman & Hall.

EIKLID, K., OLSNES, S. & PIHL, A. (1980). Entry of lethal doses of abrin, ricin, and modeccin into the cytosol of HeLa cells. *Expl Cell Res.* **126**, 321–326.

FALLON, J. F. & CROSBY, G. M. (1977). Polarizing zone activity in limb buds of amniotes. In *Vertebrate Limb and Somite Morphogenesis* (ed. D. A. Ede, J. R. Hinchliffe & M. Balls), pp. 55–69. Cambridge and London: Cambridge University Press.

GROLLMAN, A. P. (1968). Inhibition of Protein Biosynthesis V. Effects of emetine on protein and nucleic acid biosynthesis in HeLa cells. *J. biol. Chem.* **243**, 4089–4094.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

LIN, J.-Y., KAO, W.-Y., TSERNG, K.-Y., CHEN, C.-C. & TUNG, T.-C. (1970). Effect of crystalline abrin on the biosynthesis of protein, RNA and DNA in experimental tumours. *Cancer Res.* **30**, 2431–2433.

LINDELL, T. J., WEINBERG, F., MORRIS, P., ROEDER, R. J. & RUTTER, W. J. (1970). Specific inhibition of nuclear RNA polymerase II by α-amanitin. *Science* **170**, 447–450.

MACCABE, J. A. & PARKER, B. W. (1976). Polarizing activity in the developing limb of the Syrian hamster. *J. exp. Zool.* **195**, 311–317.

MONESI, V., MOLINARO, M., SPALLETTA, E. & DAVOLI, C. (1970). Effect of metabolic inhibitors on macromolecular synthesis and early development in the mouse embryo. *Expl Cell Res.* **59**, 197–206.

OLSNES, S. & PIHL, A. (1977). Abrin, ricin and their associated agglutinins. In *The Specificity and Action of Animal, Bacterial and Plant Toxins* (ed. P. Cuatrecasas), pp. 129–173. *Receptors and Recognition*, Series B, vol. 1. London: Chapman & Hall.

PERRY, R. P. & KELLEY, D. E. (1970). Inhibition of RNA synthesis by actinomycin D: Characteristic dose-response of different RNA species. *J. cell Physiol.* **76**, 127–140.

SAUNDERS, J. W. Jr. & GASSELING, M. T. (1968). Ectodermal-mesenchymal interactions in the origin of limb symmetry. In *Epithelial and Mesenchymal Interactions* (ed. R. Fleischmajer & R. E. Billington), pp. 78–97. Baltimore: Williams and Wilkins.

SAWICKI, S. G. & GODMAN, G. C. (1971). On the differential cytotoxicity of actinomycin D. *J. Cell Biol.* **50**, 746–761.

SEDMAK, J. J. & GROSSEBERG, S. E. (1977). A rapid, sensitive and versatile assay for protein using Coomasie Brilliant Blue G250. *Anal. Biochem.* **79**, 544–552.

SLATER, E. C. (1967). Application of inhibitors and uncouplers for a study of oxidative phosphorylation. *Meth. Enzymol.* **10**, 48–57.

SMITH, J. C. (1979). *Studies of positional signalling along the antero-posterior axis of the developing chick limb.* Ph.D. thesis, University of London.

SMITH, J. C. (1980). The time required for positional signalling in the chick wing bud. *J. Embryol. exp. Morph.* **60**, 321–328.

SMITH, J. C., TICKLE, C. & WOLPERT, L. (1978). Attenuation of positional signalling in the chick limb by high doses of γ-radiation. *Nature* **272**, 612–613.

SOLOTOROVSKY, M. & GABLIKS, J. (1965). The biological action of diptheria toxin in cell and tissue. In *Cell Culture in the Study of Bacterial Disease* (ed. M. Solotorovsky), pp. 5–15. New Brunswick: Rutgers University Press.

SUMMERBELL, D. (1979). The zone of polarizing activity: evidence for a role in normal chick limb morphogenesis. *J. Embryol. exp. Morph.* **50**, 217–233.

SUMMERBELL, D. & WOLPERT, L. (1973). Precision of development in chick limb morphogenesis. *Nature* **244**, 228–230.

TICKLE, C., SHELLSWELL, G., CRAWLEY, A. & WOLPERT, L. (1976). Positional signalling by mouse limb polarising region in the chick wing bud. *Nature* **259**, 396–397.

TICKLE, C., SUMMERBELL, D. & WOLPERT, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254**, 199–202.

VÁZQUEZ, D. (1979). *Inhibitors of Protein Biosynthesis.* Berlin, Heidelberg, New York: Springer-Verlag.

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