INDUCTION OF GLUTAMINE SYNTHETASE BY UREA*

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Enzyme induction in animal cells is often promoted by, indeed requires, corticosteroids (1-5). Thus far the role of the corticosteroid has not been clarified, although evidence is accumulating that it is essential for initiation of specific m-RNA synthesis if not for its translation (3,6). Recently Reif-Lehrer (7) reported an appreciable induction of glutamine synthetase (GS) on continued incubation of retinas without added corticosteroid. The mechanism of derepression of m-RNA synthesis, well understood for β-galactosidase in E. coli (8) is little understood in animal cells. It is, therefore, of interest to report derepression of the enzyme, glutamine synthetase, in the chick retina in culture by high concentrations of urea. In all likelihood this is not a natural mechanism and it is unclear to what extent other gene functions of the cell are derepressed by this treatment.

METHODS AND MATERIALS

Whole retinas were explanted to flask cultures under conditions specified by Reif-Lehrer and Amos (3). The enzyme activity was determined by the method of Waelsch (9) with modifications specified previously (3). Protein was determined by the method of Lowry et al. (10).

Actinomycin D was obtained through the courtesy of Merck and Co., Rahway, N.J.; hydrocortisone (HC) from Nutritional Biochemical Corp., Cleveland, Ohio;

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L-phenylalanine-C^{14} (sp. act. 300 mc/mM), uridine-2-C^{14} (sp. act. 44 mc/mM), from Schwarz BioResearch, Orangeburg, N.Y.; urea from Merck and Co. (Two different lot numbers were used.)

Primary cultures of chick embryo fibroblasts were prepared and cultivated as described by Amos and Moore (11). Estimates of protein content and the incorporation of radioactive amino acids and uridine into protein and ribonucleic acid (RNA) respectively were effected as reported earlier (11).

RESULTS AND DISCUSSION

Survival of Fibroblasts in 1.0 Molar Urea.

Chick embryo fibroblasts cultivated in Eagle's basal medium (BME) (12) supplemented with 3% calf serum do not multiply but synthesize protein for several days at a constant rate (11). To cells 48 hours in culture, urea dissolved in Hank's balanced salt solution (BSS) was added without change of medium to achieve concentrations varying from 0.1 M to 1.0 M. Controls received equivalent volumes of BSS. The cells showed little morphologic change after 24 or 48 hours at any concentration used. At 1.0 M urea some cells came off the surface; those remaining appeared to be quite healthy.

From the data presented in Table 1, it is evident that protein synthesis was unaffected by 1.0 M urea and was stimulated by lower concentrations of the compound. The stimulation is reflected both in the accumulation of protein during a 20-hour incubation and in the incorporation of C^{14}-phenylalaine during the same period.

In the absence of urea cells cultivated without change of medium do not multiply (11). It is as yet unclear to what extent the increase in protein synthesis on exposure to urea represents the initiation of cell replication. Both thiourea and guanidine at equivalent and lower concentrations were by microscopic examination deleterious to the cultures and inhibited protein synthesis more than 95% (unpublished data of authors). The inhibitory effect on protein synthesis was not reversed on washing the monolayer.

Derepression of glutamine synthetase (GS) in retinal cultures by urea.

Heartened by the resistance of fibroblast cultures to urea and having for some time sought means of derepressing animal cell genes non-specifically, we turned to retinal cells, to which in culture the addition of corticosteroids results in the induction of the enzyme GS (3,6). BME supplemented with 10% fetal calf serum (FCS) is generally used, since it was found to elicit little enzyme production, presumably because fetal calf serum contains little active corticosteroid.

TABLE 1

	ug protein per culture: Day 2	Day 3	Δ ug	C^{14}-Phenylalanine cpm/Mg prot/20 hrs
Expt. 1				
Control	165 ± 6	225 ± 10*	60	3300
1.0 M urea	----	240 ± 13	75	3100
0.5 M urea	----	310 ± 7	145	4500
0.3 M urea	----	435 ± 70	270 ± 70	6000
0.1 M urea	----	412 ± 12	247	6300
Expt. 2				
Control	212 ± 8	307 ± 12	95	3600
1.0 M urea	----	296 ± 9	84	4400
0.3 M urea	----	417 ± 5	205	5800
0.1 M urea	----	392 ± 20	180	6100

*Protein content expressed as average of two cultures per point. Average deviation from the mean of the two cultures

After 48 hours in culture (Day 2) the cells of two culture vessels were harvested; C^{14}-phenylalanine (0.25 μC) in BSS + urea was supplied to the other vessels which were incubated at 37°C for another 20 hours. At that time all cells were harvested, protein measured, and TCA-insoluble counts recorded.

TABLE 2

Urea Induction of GS

Treatment	Enzyme Units per 100 ug protein		
	Expt. 1	Expt. 2	Expt. 3
10% FCS "0" Time	0.3*	0.8**	0.2
10% FCS 24 hrs.	0.8	1.0	0.4
+ hydrocortisone 2 ug/ml 24 hrs.	10.8	21.4	20.5
+ 1.0 M urea 24 hrs.	0.3	0.1	---
+ 0.3 M urea 24 hrs.	4.0	12.7	---
+ 0.1 M urea "0" time	0.3	1.0	---
+ 0.1 M urea 24 hrs.	13.6	19.9	26.1
+ 0.01 M urea 24 hrs.	7.7	18.6	16.7
+ 0.001 M urea 24 hrs.	0.8	3.5	---

*Retinas from 11-day chick embryos were explanted to flasks for incubation at 37°C. Hydrocortisone or urea was present from the start. Enzyme unit = O.D. (Klett) / ug protein x 10^2. Details of culture condition (3).

**The relatively high "0" time is attributable to the 24-hour pre-incubation in FCS before addition of HC or urea.

If urea was supplied in lieu of corticosteroid (Table 2) GS was induced as well as by optimal concentrations of the corticosteroid. 0.1 M urea appeared to be most effective of the concentrations tried. The effects of the combination of urea and corticosteroid on the level of enzyme achieved are a function of sequence and timing and will be presented in a more detailed account. Again neither thiourea nor guanidine induced enzyme alone. Both caused the retinas to disintegrate and inhibited enzyme induction by corticosteroids (unpublished data of authors).

Effect of Urea on Uridine and Phenylalanine incorporation.

Whereas urea stimulated protein synthesis and C^{14}-phenylalanine incorporation

TABLE 3

C^{14}-phenylalanine and C^{14}-uridine incorporation:

Effect of Urea (0.1 M)

		^{14}C-uridine/$\frac{cpm}{mg\ prot}$		^{14}C-phenylalanine/$\frac{cpm}{mg\ prot}$	
	Time Pulse	Control	Urea	Control	Urea
Expt. 1	4 hrs.	*4,470	4,650	703	677
	24 hrs.	17,980	17,070	2,910	2,720
Expt. 2	4 hrs.	4,240	4,200		
	24 hrs.	16,490	16,430		

*Retinas incubated 24 hours at 37° C in BME plus 10% FCS before simultaneous addition of radioactive precursor, and urea (0.1 M) where added. Cold TCA-ppt cpm/mg protein. All values reported are average of two retinas separately processed. (No members of a pair deviated from the mean more than 6%.)

in fibroblast cultures, in retinal cells no effect of 0.1 M urea was observed for short (4-hour) or long (24-hour) pulses of C^{14}-phenylalanine or C^{14}-uridine (Table 3). Since there is no accumulation of RNA or protein in retinal cells in short-term culture, incorporation of precursors is the only way to estimate macromolecule synthesis.

Kinetics of Induction of GS by HC or Urea.

Comparison of the kinetics of induction by 0.1 M urea and 2 µg/ml HC produced little evidence of difference in appearance of enzyme (Fig. 1).

Effect of Act. D and of Pulse of Urea.

As with HC- induced enzyme urea induction was inhibited by actinomycin (1 ug/ml or 5 ug/ml) (Table 4) when the drug was supplied at the start. This result can be taken as presumptive evidence that RNA synthesis is required.

The observation that cells washed free of urea continue to synthesize enzyme about as well for at least 20 additional hours cannot be interpreted at

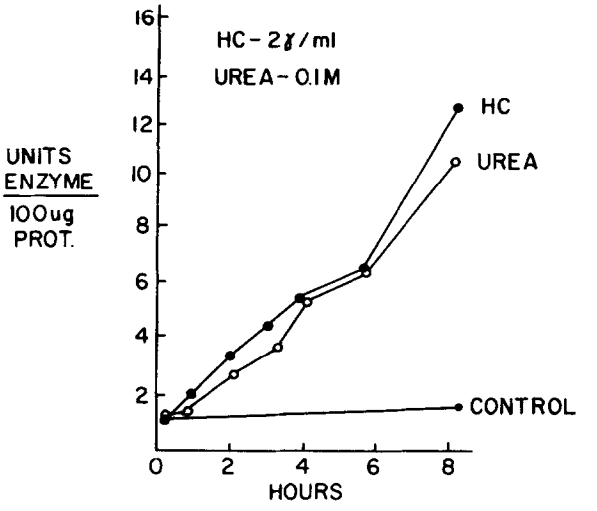
present. It is possible that urea bound intracellularly cannot be washed out easily. Evidence presented in Table 2 suggests that concentrations of urea as high as 0.01 M are not as effective over 24 hours as 0.1 M.

TABLE 4

Prevention of Induction by Act. D: Effect of 4-hour "Pulse" of Urea

	Enzyme units/100 ug protein	
	Time	
Expt. 1	4 hrs.	24 hrs.
No HC	----	0.7
HC (2 ug/ml)	----	15.2
+ Act. D (1 ug/ml)	----	0.5
+ Act. D (5 ug/ml)	----	0.6
Urea (0.1 M)	----	15.3
+ Act. D (1 ug/ml)	----	0.7
+ Act. D (5 ug/ml)	----	0.8
Expt. 2		
HC (.02 ug/ml)	2.5	16.0
washed 4 hrs.	2.5	6.5
Urea (0.1 M)	2.3	20.4
washed 4 hrs.	2.3	16.2

Retinas were incubated for 24 hours in BME plus 10% FCS before start of experiment. In experiment 2 all retinas were supplied at "zero" time with either HC or urea. After 4 hours incubation some retinas were harvested; all others were washed twice with BSS, and returned to fresh medium with or without the inducer with which they were incubated for 4 hours.



Legend to Figure 1

Comparison of Induction of GS by HC and Urea

Retinas were incubated at 37° C for 24 hours before introduction of HC or urea. Cells from two retinas were harvested and processed separately for each point. Enzyme was assayed as described (3).

Comment

Obviously the question of principal interest in this work is the mechanism of derepression by urea, a derivative of urea or a contaminant in the urea*. Neither ammonia nor hydroxylamine, nor any of the 20 usual amino acids can be shown to affect enzyme induction in the presence or absence of corticosteroids (unpublished data of the laboratory).

Of equal interest is the question of the extent of the derepression of the cell genome. The expanded protein synthesis observed with fibroblasts indicates that multiple effects of urea can be anticipated.

*In an effort to eliminate a contaminant molecule, the urea was recrystallized sequentially twice from a saturated solution at 60°-70° C by cooling to 0°C and twice from ethanol by the same procedure before being run over a mixed bed AG501-8XLD resin (Bio-RAD). It was then sterilized by filtration before addition to the culture medium. Biuret positive material less than 1 part in 10^4 (by weight). The inducing activity of the recrystallized material is the same as the starting urea.

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