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Biochemical Effects of Cycloheximide in Developing Chick Brain

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WOOLSTON, M. E., I. G. MORGAN AND J. W. HAMBLEY. *Biochemical effects of cycloheximide in developing chick brain.* PHARMAC. BIOCHEM. BEHAV. 10(2) 245–249, 1979.—The timecourses of inhibition of protein synthesis in forebrain roof 15 min to 24 hr after either intracerebral or peripheral injection of 40 μg of cycloheximide (CXM), show maximum inhibition (68–85%) 1 hr after injection in 12 hr, 2-day- and 16-day-old chicks. In 2-day-old chicks, the level of free lysine was elevated by around 250% 1 hr after intracerebral injection of CXM. The total radioactivity in the forebrain roof/mg protein following peripheral injection of labelled lysine increased by around 80% compared to saline controls. Following unilateral injection of 20 μg in 25 μl CXM into a forebrain hemisphere, the inhibition of protein synthesis spread to the opposite forebrain hemisphere and both optic lobes within 5 min. Smaller volumes did not seem to give a clear unilateral inhibition capable of explaining the unilateral behavioural differences which have been reported. Central injection of CXM in 2- and 16-day-old chicks, resulted in an inhibition of liver protein synthesis of 55% and 40%, respectively. There were also gross signs of pathological effects in the liver and gall bladder. The implications of these effects of CXM are discussed in terms of the use of the drug in behavioural experiments.

Brain Protein synthesis Lysine Cycloheximide

CYCLOHEXIMIDE (CXM), which inhibits cytoplasmic ribosomal protein synthesis in eukaryocytes, has been widely used in a number of experimental situations to produce amnesia in a range of species [10]. In addition it has been reported to produce behavioural retardation [16], increased persistence [14] and to mediate conditioned aversions [3] in chickens. Important questions arise when attempting to correlate this variety of behavioural effects with inhibition of cerebral protein synthesis. For example, for any given route of administration, are there pronounced inhibitions of protein synthesis occurring in remote tissues? For how long is inhibition maintained? For studies in which developmental differences are apparent, is the drug equally effective at different ages?

The neonatal chick is a convenient animal for many learning experiments. It can be readily imprinted, performs well in passive avoidance tasks and moreover has a poorly developed blood-brain barrier [14] which facilitates the use of radiolabelled metabolic precursors. To provide base-line information for studies of the type described by Bolas *et al.* [4] and for studies in our own group, we have studied the time course of inhibition of protein synthesis in forebrain roof after peripheral and intracerebral injection of CXM in chickens 12 hr, 2 days and 16 days after hatching. The degree of inhibition in each side of the forebrain following unilateral intra-cerebral injection of CXM was also examined, as was the effect of intra-cerebral CXM on liver protein synthesis and cerebral free lysine levels.

GENERAL METHOD

White Leghorn × Black Australorp chicks from Research Poultry, Victoria, Australia, were hatched and maintained in our laboratory with food and water ad lib. Peripheral injections of 40 μg CXM in 50 μl saline were made into the pericardial cavity. Central injections of 20 μg CXM in 25 μl saline were placed free-hand into each forebrain hemisphere, using a rubber stop on the 0.45×13 mm needle to ensure that it did not penetrate more than 3 mm below the surface of the skull. An equal volume of 0.9% saline was injected either centrally or peripherally in control animals. After the appropriate time interval, 2.5 μCi ^{14}C-lysine monohydrocloride in 100 μl was injected into the pericardial cavity. At specified times after injection, animals were decapitated. In Experiments 1 and 2, forebrain roofs were removed (as described by Horn *et al.* [12]) and homogenized. In Experiment 3, whole forebrains and optic lobes were removed.

The total radioactivity was determined by solublizing an aliquot of the homogenate in 1 ml Protosol (New England Nuclear) tissue solublizer. As lysine is only slightly, if at all, metabolized into non-protein material [7], lysine incorporation into TCA-precipitatable material is taken as an index of protein synthesis. The radioactivity incorporated into protein was determined by precipitation with 10% trichloracetic acid (TCA) containing 10 mM lysine, and centrifugation at 1000 G for 10 min. Pellets were then resuspended in TCA containing 10 mM lysine and washed 3 times. The pellets were then digested in 1 ml Protosol. All samples were

counted in a Beckman LS-250 liquid scintillation counter. The relative specific activity (RSA) is the ratio of TCA precipitated radioactivity to the total radioactivity. The percentage inhibition is the difference between the saline RSA and the CXM-RSA as a percent of the saline RSA, i.e., % inhibition $=100\times(RSA_{SAL}-RSA_{CXM})/RSA_{SAL}$. Each percentage inhibition represents at least 5 saline chicks and 5 CXM chicks.

For amino acid analysis, forebrains were homogenized in 10% (w/v) sulphosalicylic acid (SSA). After standing on ice for 30 min the precipitate was removed by centrifugation and the amino acid composition of aliquots of the supernatant was measured by an automated column chromatographic procedure.

EXPERIMENT 1

To determine the time of maximum inhibition and the recovery time of protein synthesis, lysine incorporation was measured after intervals of time between 15 min and 24 hr following central of peripheral CXM in 12 hr, 2 day and 16 day-old chicks.

Results

Figures 1 and 2 show that maximum inhibition occurred at around 1 hr after injection, regardless of age or route of injection. The inhibition by central CXM in all ages and peripheral CXM in 12 hr old chicks followed the same course up to 6 hr, while peripheral CXM inhibition in 2- and 16-day chicks dropped rapidly after the peak. The onset of inhibition of protein synthesis after pericardial injection of CXM was significantly ($p<0.05$) slower in 16-day old birds than in the other two groups. Protein synthesis in 2- and 16-day-old chicks recovered (and overshot normal rates in some cases) by 24 hr, while the inhibitory effects were still apparent in 12-hr-old chicks. The stimulation of protein synthesis is significant ($p<0.025$) only in the 16-day birds.

Discussion

According to these results, the relevant behavioural manipulations ought to be performed around 1 hr after injection of CXM in order to coincide with the maximum drug effect. However, the related drug, acetoxycycloheximide can effect memory when injected as much as 5 hr before training [6,17]. Moreover, CXM has been reported to be effective when injected after learning [13]. The important point is that for each animal and behavioural situation there will be a different time course for the sequels of the behavioural manipulation. Where we do not have precise details of this sequence, an empirical study of the time-course of CXM's effect is the best approach. Bearing this in mind, we have found that in young chickens, there is appreciable inhibition of protein synthesis for up to 6 hr after injection, which would seem to set an upper limit to the drug-experience delay.

Peripheral injection of CXM gives equivalent results to those obtained with intracerebral injection only during the first day after hatch, but it has the obvious advantage of not requiring physical disruption of the brain. The decreasing effect of peripherally-injected CXM with age could be explained by dilution due to increasing body weight, the development of a blood brain barrier, or to a decreased sensitivity of brain protein synthesis to the drug. The fact that at all ages intra-cerebral CXM has similar effects rules out the last possibility. While it is known that blood-brain barriers develop in the young chick over the period studied, we cannot conclude from our results that this is the explanation.

EXPERIMENT 2

To investigate the effects of CXM on free lysine concentrations 2-day-old birds were killed 1 hr after either CXM or saline intra-cerebral injections. Lysine concentrations were determined as described in General Method. In a parallel experiment the total radioactivity in the forebrain of CXM-injected birds was compared with that of saline-injected

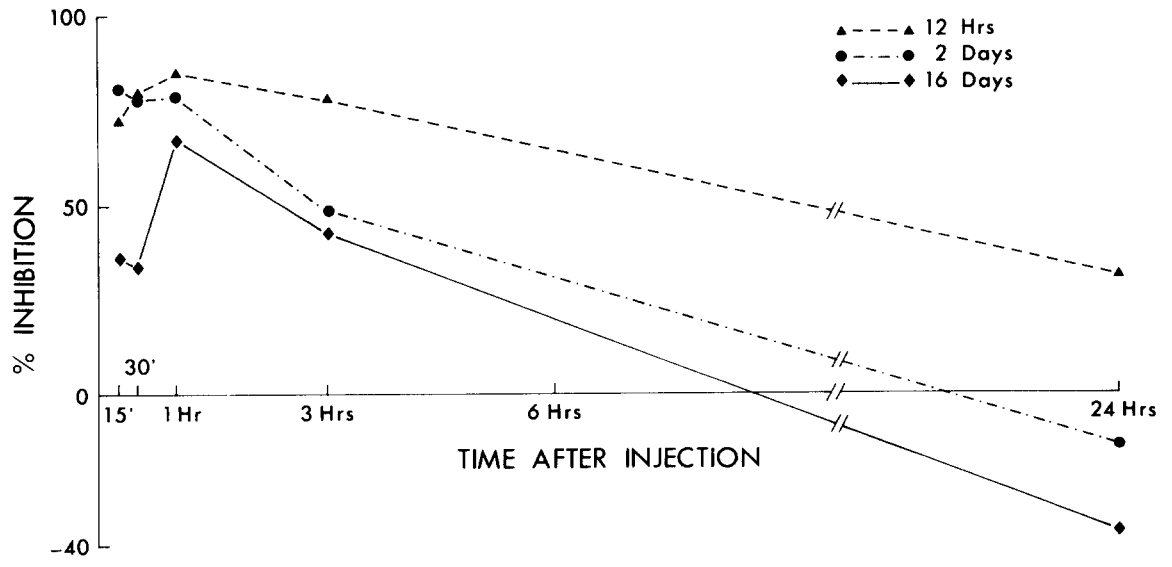

FIG. 1. Time-course of inhibition of protein synthesis by peripherally administered cycloheximide (40 μg/40 μl) in 12 hr, 2-day- and 16-day-old chickens. ^{14}C-lysine was injected pericardially.

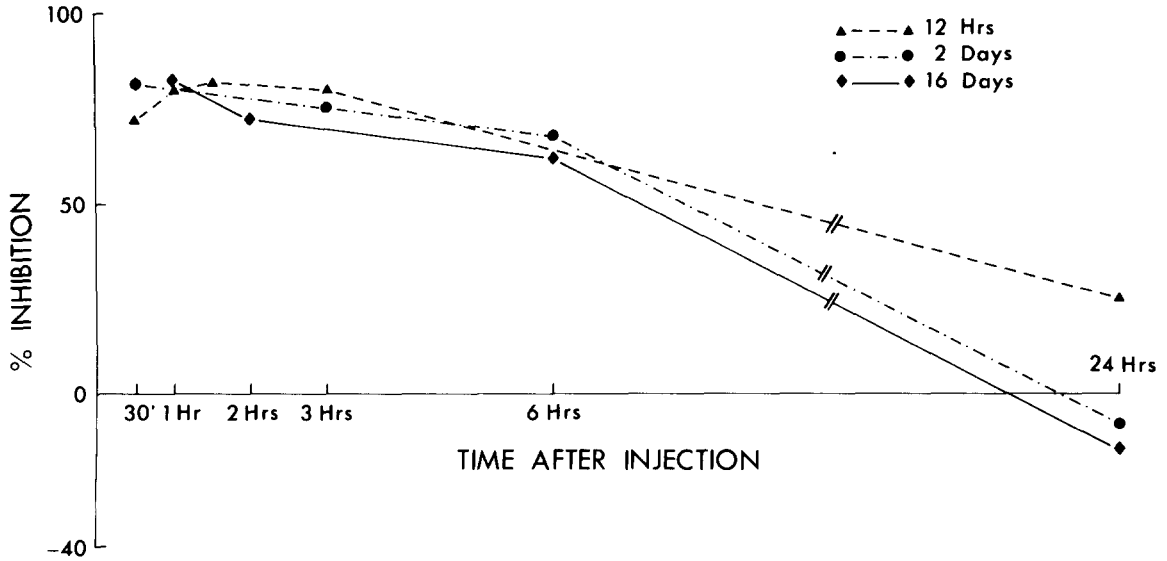

FIG. 2. Time-course of inhibition of protein synthesis by intracerebrally administered cycloheximide(40 μg/40 μl) in 12-hr, 2-day- and 16-day-old chickens. ^{14}C-lysine was injected pericardially.

birds. The time between injection of CXM or saline and amino acid injection was 1 hr, with a lysine incorporation time of 20 min. Other procedures were as described in the General Method.

Results

The results are shown in Table 1. Lysine concentrations after 1 hr of CXM were elevated by 257% compared to saline controls. The total radioactivity increase after CXM treatment was 82% in this experiment.

Discussion

We have shown in this experiment that at a time when protein synthesis inhibition is maximum, there is a marked elevation in free lysine pool. There is a similar increase in total labelled lysine 1 hr after CXM injection. The increase in labelled lysine could be a reflection of the brain becoming more permeable to blood-borne lysine (all lysine injections are pericardiac), in the same way that higher doses of CXM appear to disrupt the blood-brain barrier in rats [1]. Alternatively the increase could reflect increased exchange of labelled blood lysine with the enlarged brain pool of lysine which occurs following the inhibition of protein synthesis while protein degradation continues. Whatever the explanation, this represents another complicating factor in the drug's effects on cerebral metabolism. The observed increase in total and labelled lysine seen after 1 hr may have led us to a false estimate of the degree of inhibition of protein synthesis. In calculating the degree of inhibition of protein synthesis, it is always assumed that there is no change in the precursor pool size. Calculating relative specific activities then compensates for changes in specific activity of the pool. However, these results suggest that CXM in fact changes the size of the pool as well. No other workers, to our knowledge, have even considered this possibility. But the problem is at the moment intractable as we do not know in which cellular compartments these increases occur. For more detailed studies of brain protein synthesis precise estimation of the specific activity of the real precursor, lys t-RNA, might be necessary.

TABLE 1

LYSINE CONCENTRATIONS AND TOTAL RADIOACTIVITY PER FOREBRAIN (±SEM) 1 HOUR AFTER CXM OR SALINE INTRACEREBRAL INJECTIONS. CONTROL AND EXPERIMENTAL GROUPS CONTAINED AT LEAST 5 ANIMALS

	Lysine p mol/mg wet wt	Total Radioactivity per Forebrain cpm/mg Protein
CXM	666.4 ± 52.9	1105.8 ± 142.8
Saline	186.2 ± 20.7	607.6 ± 81.6
% increase	257%	82%

In addition the free pools of other physiological amino acids were increased after CXM and this too may pose problems for CXM-induced behavioural effects, for in certain studies the accumulation of free amino acids may be at least as important as the inhibition of protein synthesis *per se* [11]

EXPERIMENT 3

Several workers have reported that unilateral injection of CXM produces different effects to bilateral injections, and that unilateral injection can produce unilateral behavioural lesions [2,15]. The relatively high degree of protein synthesis inhibition observed in our experiments suggests that CXM is effective throughout the hemispheres. Therefore two questions were asked. Does cycloheximide inhibit areas of the brain other than the forebrain, and does unilateral injection of CXM result in localized inhibition of protein synthesis?

TABLE 2

INHIBITION OF PROTEIN SYNTHESIS FOLLOWING UNILATERAL INJECTION OF VARIOUS DOSES OF CXM, COMPARED TO CONTROLS INJECTED WITH SALINE. CONTROL AND EXPERIMENTAL GROUPS CONSISTED OF AT LEAST 5 ANIMALS EACH

Experiment	Forebrain Hemisphere Injected	Amount of CXM (μg)/Volume (μl)	Drug-Amino Acid Interval (min)	Pulse Length (min)	% Inhibition of Protein Synthesis			
					Left Forebrain	Right Forebrain	Left Optic Lobe	Right Optic Lobe
3a	Left	40/25	60	20	86.2	81.6	82.0	82.2
3b	Left	20/25	15	20	44.6	36.1	—	—
	Right	20/25	15	20	43.2	45.1	—	—
3c	Left	20/25	5	10	62.8	59.3	60.5	55.7
3d	Left	5/5	5	10	38.3	36.7	24.3	25.6

*At no stage comparing injected and non-injected forebrain hemispheres, or right and left lobes was there a statistically significant ($p<0.01$) difference between the R.S.A.'s using Student's t-test.

The possibilities were tested by injecting 20 μg CXM in 25 μl into one hemisphere and following the rates of protein synthesis after 1 hr. In view of the general high degree of inhibition of protein synthesis observed, we then injected lesser amounts and volumes in order to see if the inhibition of protein synthesis was localized under these conditions.

Results

Table 2 shows that at a dose of 40 μg CXM in 25 μl injected unilaterally into 2-day old chick forebrain, there was a high degree of inhibition of protein synthesis not only in the injected forebrain and in the saline-injected forebrain, but also in the two optic lobes. The degree of inhibition was the same in all areas.

Since the high degree of inhibition could have resulted in threshold effects, we then varied the volume injected, the dose and the time between drug and amino acid injection, in order to minimise diffusion of the drug. While the degree of protein synthesis inhibition was reduced, there was still no evidence for even a slightly preferential inhibition of protein synthesis in the injected forebrain hemisphere as early as 15 and even 5 min after injection.

In a final attempt to reduce the spread of the inhibitory effect of CXM, we reduced the dosage to 5 μg of CXM in 5 μl. Even under these conditions, there was no evidence for preferential inhibition of protein synthesis in the injected forebrain hemisphere as early as 5 min after injection.

Discussion

These results show clearly that within a very short period after injection of the drug, probably as soon as 5 min after, CXM appeared not only to have reached, but to be exerting its inhibitory effect all over the brain.

The shortest drug-amino acid interval was 5 min, followed by a 10 min pulse, but even under these conditions, with the lowest volume (5 μl) used, the drug was exerting an effect in all the brain areas studied. If localised injections of drug are required, it will probably be necessary to use even lower volumes in order to limit the spread.

The results make it very difficult to interpret the unilateral inhibitions of memory formation reported by Bell and Gibbs [2]. They used a slightly higher dose (10 μg/10 μl) than our lowest dose and this should have resulted in inhibition of protein synthesis all over the brain. The inhibition of protein synthesis should have reached a maximum within the time after training normally found to be necessary for amnesia on the one-trial passive avoidance task used. However, the birds used in this study had been unilaterally trained before injection, and this may in some way have resulted in an asymmetry which we did not observe in our experiments. Blood flow changes are known to occur as a result of learning [5] and further studies are in progress to investigate this possibility.

EXPERIMENT 4

As well as the cerebral metabolic effects of CXM, there may be significant peripheral effects, even following central injection, which could be associated with reported aversive effects of CXM [4]. To test this, 40 μg CXM in 50 μl was injected centrally and the amount of protein synthesis in the livers of 2- and 16-day-old chicks was measured 1 hr after injection.

Results

Centrally injected CXM resulted in 55% inhibition of liver protein synthesis in 2-day-olds and 40% in 16-day-old chickens. It was also noted that CXM-treated chicks had distended gall bladders 1 hr after injection, and livers appeared blotchy in birds killed longer than 1 hr after CXM injection.

Discussion

The considerable effect of cerebral injection of CXM on body organ protein synthesis and evidence of gross pathological changes is a further complication in the interpretation of behavioural effects. Factors such as motivation and performance must be carefully considered and controlled if doses of CXM which are necessary for behavioural effects result in sickness. The contention of Bolas *et al.* [4] that intracerebral CXM can be aversive in conditioned avoidance experiments is certainly not at odds with the observed inhibition of liver protein synthesis which may be associated with gastro-intestinal upset.

GENERAL DISCUSSION

In addition to the possible direct side-effects of CXM,

there are effects resulting from the protein synthesis inhibition caused by CXM. These "flow-on" effects could include phenomena such as blotchy liver, distended gall bladder, decrease in catecholamine levels [18], increase in amino acid levels [11], etc. Such "flow-on" effects cannot be controlled for by using a variety of other protein synthesis inhibitors, since it is the inhibition of ribosomal protein synthesis itself which triggers off these processes. There is of course another class of sequels which is indirect, but drug-specific, such as the toxic effects of peptidyl-puromycin [7].

The claimed advantages and disadvantages of the two modes of injection [9] are not clear-cut. Peripheral injection of CXM is not necessary in order to produce wide-spread inhibition of protein synthesis in brain, for at least with the doses and volumes used in these studies, central injection did not result in localized inhibition. Conversely, central injection of CXM does not rule out inhibition of protein synthesis in the periphery.

There is no logical reason to expect that the biochemical and behavioural consequences of CXM we have measured should correspond in time, given the crudity of the behavioural and biochemical measures used in all work. We do not know in which area of the brain the relevant inhibition of protein synthesis and the ultimate effect of lack of new proteins is exerted, nor in which cellular compartment the latter occurs. Similarly, we do not know at what stage in the sequence of effects of a behavioural experience, inhibition of protein synthesis might be important.

However, our results do establish that both for peripheral and central injections of CXM, the maximum inhibition of protein synthesis is produced about 1 hr after injection. Greater than 50% inhibition of protein synthesis lasts for 6–12 hr. In the absence of more detailed information this would suggest that an interval of 1 hr between injection of CXM and the behavioural event under study should be optimum, although the possibility of CXM-induced illness at this time and the anorexia observed by Bolas *et al.* [4] obviously needs to be taken into account.

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