We demonstrate here that ethanol, in contrast to heat shock (Chousterman, S., Chelbi-Alix, M. K., and Thang, M. N. (1987) J. Biol. Chem. 262, 4806–4811), induces interferon (IFN) synthesis and its related activities in Madin-Darby bovine kidney (MDBK) cells. The induced IFN is secreted maximally at 6 h, whereas the induction of 2′,5′-oligoadenylate synthetase mRNA peaks between 9 and 12 h and its activity at 15 h. The appearance of both 2′,5′-oligoadenylate synthetase activity and the antiviral state upon ethanol treatment is prevented by anti-bovine recombinant IFN-β antibodies. Bovine diarrhea virus infection-free MDBK cells cultured in medium supplemented with serum substitute also gave similar results, thus indicating that IFN synthesis induced by ethanol is not mediated by the activation of bovine diarrhea virus.

Together, these results show that: 1) ethanol induces the 2′,5′-oligoadenylate synthetase and antiviral activities through IFN-β production; and 2) the IFN produced does not act directly from inside the cells, but has to be first secreted to bind to its receptor. In MDBK cells, ethanol induces the synthesis of the 70-kDa protein, which precedes the expression of 2′,5′-oligoadenylate synthetase; moreover, the transient nature of the synthesis of the hsp 70 in these cells is similar after both heat shock and ethanol treatment.

In all organisms examined so far, hyperthermia induces the synthesis of a set of proteins known as heat shock or stress proteins (hsp) (1–3). Many other inducers of hsp are also effective across a broad range of species (4, 5). Thus, ethanol induces hsp in Escherichia coli (6), yeast (7, 8), and mammalian cells (9). Much attention has been devoted to the mechanism of induction of hsp (3–5); however, little is known about the events occurring during the recovery period, i.e. after return from stress to normal growth conditions (10, 11), and, in particular, about the mechanisms involved in the turnover of hsp and their mRNAs.

The 2′,5′-oligoadenylates are known to be potent inhibitors of protein synthesis through the degradation of mRNA by activating a latent endoribonuclease (12). The 2′,5′-oligoadenylate synthetase (2′-5′A synthetase), after being activated by double-stranded RNA, catalyzes the synthesis, from ATP, of the unusual oligonucleotides linked by 2′,5′-phosphodiester bonds (13). 2′-5′A synthetase expression is usually induced in interferon (IFN)-treated cells (14); but various substances other than IFN, such as corticoid hormones (15), butyric acid and dimethyl sulfoxide (16), nerve growth factor (17), and platelet-derived growth factor (18), can also induce this enzyme.

We have recently observed that 2′-5′A synthetase is implicated in the cell response to hyperthermia (19); thus, heat-shocked MDBK cells showed a 25-fold increase in 2′-5′A synthetase activity during the recovery period. Moreover, heat-shocked MDBK cells released into the medium a heat shock-induced factor capable of inducing an increase in 2′-5′A synthetase activity in nonstressed cells; heat shock-induced factor is different from IFN-α, -β, or -γ as demonstrated by the lack of reaction of anti-bovine IFN antibodies (19). Using another stress, i.e. ethanol treatment, we examined whether or not the same mechanism as that observed after heat shock is involved during cell recovery.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Electrophoretic Pattern of Proteins Synthesized during Ethanol Treatment and Subsequent Recovery—The synthesis of a set of specific proteins in response to stress has been described in various mammalian cells (2). Since ethanol is also a stress agent (4, 5), we studied, in MDBK cells, the proteins synthesized after ethanol treatment and compared them with those induced after heat shock (19). Cell proteins were pulse-labeled for 1 h with [35S]methionine in untreated and ethanol-treated cells at various times (from 1 to 18 h) during the recovery period (during incubation at 37 °C with ethanol-free medium, after removing ethanol). The proteins in the different extracts were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and labeled proteins were detected by autoradiography (Fig. 1). As observed in MDBK cells (19) and in other cell types submitted to heat shock (30), normal protein synthesis was reduced by 70% in MDBK cells after 1 h of ethanol treatment (data not shown).

The concentration of ethanol chosen for these studies (7.5%, v/v) was derived from the experiments described below. Exposure of the cells to 7.5% (v/v) ethanol for 1 h at 37 °C

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*This work was supported by Centre National de la Recherche Scientifique Grant UAC 115 and Institut National de la Santé et de la Recherche Médicale Grant U-245. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Abbreviations used**: hsp, heat shock protein(s); IFN, interferon; rIFN, recombinant IFN; MDBK, Madin-Darby bovine kidney; 2′-5′A synthetase, 2′,5′-oligoadenylate synthetase; VSV, vesicular stomatitis virus; BDV, bovine diarrhea virus; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
induced the synthesis of a typical set of proteins known as stress proteins. The analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1) revealed the synthesis of the 70,000-dalton protein(s), one of the major and highly conserved proteins in most organisms (31) including MDBK cells (19). The synthesis of hsp 70 was already detectable after 1 h of ethanol treatment (lane 1); during the recovery period, it increased between 1 and 2 h (lane 2), reached a maximum between 3 and 4 h (lane 3), declined between 5 and 6 h (lane 4), and disappeared after 9 h (lanes 5 and 6). Thus, in MDBK cells, the kinetics of the appearance and disappearance of hsp 70 are similar after heat shock (19) and ethanol treatment.

Effect of Ethanol on Cell Growth—To determine the effect of ethanol on cell proliferation, MDBK cells were treated with 7.5% (v/v) ethanol at 37°C for 1 h and then washed and incubated at 37°C in ethanol-free medium for recovery; the cell numbers were counted at various times during recovery as indicated in Fig. 2. At the time of removal from ethanol-containing medium, 20% of the cells (compared to untreated cells) were detached and thus were lost (Fig. 2), whereas the rest showed an altered morphology that returned to normal soon after transfer to ethanol-free medium. After the removal of ethanol, growth recovered rapidly during the first 4 h and then became normal. Both treated and untreated cultures reached stationary phase at 40 h; at this stage, the population of the ethanol-treated cells was only 7% less than that of the untreated cells. Thus, after recovery from ethanol treatment, the growth rate was significantly enhanced (Fig. 2); and by trypan blue test, almost 99% of the cells were viable under both conditions (data not shown).

Increase in 2′-5′A Synthetase Activity during Recovery after Ethanol Treatment—The basal level of 2′-5′A synthetase activity did not change in MDBK cells treated with 7.5% (v/v) ethanol for 1 h at 37°C. Moreover, as in the case of heat shock (19), 2′-5′A synthetase activity increased only during the recovery period. The kinetics of this increase during recovery (Fig. 3) showed that significant enhancement started 6–9 h after the removal of ethanol, reached its maximum at 15 h, and then declined slowly; 50% of the enzyme activity was still present after 24 h.

To determine the optimum concentration of ethanol and the time period required for a maximum increase in 2′-5′A synthetase activity during recovery, MDBK cells were incubated at 37°C with different concentrations of ethanol for 1 h (Fig. 4A) or with 7.5% (v/v) ethanol for various time periods (Fig. 4B). The cells were then washed and incubated with ethanol-free medium at 37°C, and enzyme activity was measured 17 h later.

The results show that no significant increase in 2′-5′A synthetase activity occurred with ethanol at concentrations between 2.5 and 5% (v/v) (Fig. 4A); at 7.5%, however, the increase reached its maximum between 60 and 80 min (Fig. 4B) with a specific activity corresponding to 25 times the basal level. This maximum increase was observed at 8%; but at higher concentrations of ethanol, induced 2′-5′A synthetase activity diminished and corresponded to 87, 76, and 53% of the maximum at 8.5, 9, and 10% (v/v) ethanol, respectively (Fig. 4B). With 10% (v/v) ethanol, this maximum was reached at 30 min (data not shown). We chose to use 7.5% (v/v) ethanol for 1 h in all these studies.

In human WISH cells also, ethanol increased 2′-5′A synthetase activity during the recovery period. The maximum increase was obtained with a concentration of 6% (v/v) ethanol for 10 min. This maximum was almost the same as that in ethanol-treated MDBK cells (154 versus 130 units/mg of protein) and corresponded to only a 2.3-fold increase over the basal level. This small relative increase could be attributed to the high endogenous 2′-5′A synthetase activity in untreated WISH cells.

Induction of 2′-5′A Synthetase mRNA by Ethanol in MDBK cells—To obtain independent evidence for the induction of 2′-5′A synthetase by ethanol in MDBK cells, total RNA extracted from these cells was subjected to Northern blot analysis and probed for 2′-5′A synthetase mRNA (Fig. 5). MDBK cells were treated for 1 h with 7.5% (v/v) ethanol at 37°C and then shifted to ethanol-free medium for recovery. Cytoplasmic RNA was isolated from the cells at different periods, and identical amounts were electrophoresed for Northern blot analysis (Fig. 5). In untreated MDBK cells, 2′-5′A synthetase mRNA was undetectable (lane 1). In cells recovering from ethanol treatment, the presence of 2′-5′A synthetase mRNA became detectable at 6 h (data not shown), increased to a maximum at 9 h (lane 2), remained at the same level at 12 h (lane 3) and declined after 15 h (lane 4).

Induction of Antiviral State in Parallel to Increase of 2′-5′A Synthetase Activity in Ethanol-treated Cells—Since we observed, in cells recovering from ethanol treatment, an increase in both 2′-5′A synthetase mRNA and activity, it was of interest to determine if these cells established an antiviral state when infected with vesicular stomatitis virus (VSV). As expected, bovine rIFN-β induced antiviral and 2′-5′A synthetase activities in MDBK cells. These two induced activities were abolished in the presence of anti-bovine rIFN-β antibodies (Table IA). Table IB shows that ethanol-treated MDBK cells developed an antiviral state and that the inhibition of VSV yield (200 times less than control infected cells) paralleled the increase in 2′-5′A synthetase activity. Induction of enzyme activity after ethanol treatment required RNA and protein synthesis since it was abolished by actinomycin D and cycloheximide (Table IC). This shows clearly that 2′-5′A synthetase activity cannot be directly activated by ethanol. Since, so far, only IFN has been described as specific inducer of both 2′-5′A synthetase and antiviral activities, it was important to determine whether these inductions were due to an IFN-like intermediate. Therefore, we studied the effect of anti-bovine rIFN antibodies on the 2′-5′A synthetase and antiviral activities induced in the ethanol-treated MDBK cells. For this purpose, MDBK cells were treated with anti-bovine rIFN antibodies during ethanol treatment and the recovery period. Table IB shows that these two induced activities were abolished by the presence of anti-bovine rIFN-β, but not by that of anti-bovine rIFN-α or γ antibodies (in each case, the amounts used were sufficient to neutralize 500 units of IFN). These results suggest that the factor(s) inducing both 2′-5′A synthetase and antiviral activities could be IFN-β and that it exists in the culture medium of ethanol-treated MDBK cells.

Characterization by Antibody Cross-reactions of Ethanol-induced IFN—The concentration of the ethanol-induced factor, if present in the culture medium, could be measured by the capacity of the cell-free culture medium to induce 2′-5′A synthetase activity and to inhibit VSV yield in untreated MDBK cells. As shown in Fig. 6, this factor accumulated during the first hours of the recovery period, and its enzyme activity-inducing capacity reached a maximum when cells were allowed to recover for 4–6 h and then diminished. When the culture medium from ethanol-treated cells after 6 h of recovery was tested for its capacity to inhibit VSV yield, it showed an antiviral activity corresponding to 15 IU/ml IFN (Fig. 6). This IFN titer value declined after 8 h of recovery and disappeared after 11 h. As illustrated in Fig. 6, this IFN
induced 2'-5'A synthetase and antiviral activities with roughly similar kinetics. To confirm that ethanol-induced IFN is IFN-β, the culture medium from ethanol-treated cells after 6 h of recovery, which contained ethanol-induced IFN, was treated with different anti-bovine rIFN antibodies and then transferred to nonstressed MDBK cells. Table II shows that the 2'-5'A synthetase and antiviral activities induced by ethanol-induced IFN were abolished by anti-bovine rIFN β antibodies, whereas anti-bovine rIFN-α or -γ antibodies had no effect on the induction of 2'-5'A synthetase activity. These results demonstrate that in ethanol-treated MDBK cells, the induction of 2'-5'A synthetase and the establishment of the antiviral state are mediated through the induction followed by the secretion of IFN-β.

Although it is known that MDBK cells are susceptible to infection with bovine diarrhea virus (BDV), the observed production of IFN-β is, however, not due to an activation of virus by ethanol because the same results were obtained with MDBK or BDV infection-free MDBK cells (Figs. 4 and 6 and Tables I and II). We have also verified that the production of IFN-β was not due to ethanol activation of some component in the serum or in the serum substitute (basal medium supplement) since ethanol was also capable of inducing IFN-β in MDBK or BDV infection-free MDBK cells incubated with nonsupplemented medium during ethanol treatment and the recovery period (data not shown).

**DISCUSSION**

It is known that ethanol induces stress proteins in yeast (6, 7) and mammalian cells (8). In this work, we have demonstrated that in MDBK cells, ethanol, like heat shock (19), induces the synthesis of the 70-kDa protein, one of the highly conserved stress proteins. The appearance and disappearance of hsp 70 in these cells follow similar kinetics after ethanol treatment or heat shock. The synthesis of hsp 70, already detectable during ethanol treatment, attains a maximum between 3 and 4 h during recovery, declines between 5 and 6 h, and then disappears after 9 h.

We have also shown that ethanol induces 2'-5'A synthetase activity in MDBK cells. This induction is, however, not an early event, but occurs much later during the recovery period since the enhancement was significant only 6–9 h after the removal of ethanol and reached a maximum at 15 h. The kinetics of the induction of 2'-5'A synthetase after heat or ethanol treatment are similar during the first 15 h of recovery and then differ significantly; however, 2'-5'A synthetase activity induced after heat treatment remained stable for at least 25 h (Fig. 1 in Ref. 19), whereas that induced after ethanol treatment decreased to <50% of the maximum at 24 h. The comparison of these kinetics, both after heat shock (19) or after ethanol treatment, shows clearly that the synthesis of hsp 70 precedes the expression of 2'-5'A synthetase. The induction of 2'-5'A synthetase by ethanol was also confirmed by Northern blot analysis of its mRNA; during recovery, at 6 h, there was only a small increase of this mRNA. This increase reached a maximum at 9 h, remained at the same level at 12 h, and then decreased after 15 h.

The maximum increase in 2'-5'A synthetase activity after ethanol treatment corresponds to 25-fold of the basal level in MDBK cells. Under the same conditions (7.5% ethanol for 1 h), no such enhancement was observed in 2'-5'A synthetase activity in human WISH cells. However, a significant increase (2.3-fold over the basal level) was obtained after treatment with 6% (v/v) ethanol for 10 min. This is because endogenous 2'-5'A synthetase activity is already very high in untreated WISH cells. Ethanol-treated MDBK cells developed an antiviral state when infected with VSV; moreover, the anti-bovine rIFN-β antibodies abolished both the 2'-5'A synthetase and antiviral activities induced after ethanol treatment.

The enhancement of 2'-5'A synthetase activity after ethanol treatment was, like that after heat shock (32, 33), dependent on both RNA and protein synthesis. The increase in 2'-5'A synthetase expression after ethanol treatment must be expressed as a secondary response to the stress since: 1) 2'-5'A synthetase mRNA and activity increased much later than the maximum synthesis of hsp 70; and 2) the culture medium after recovery from ethanol-treated cells has the capacity to induce both the enzyme and antiviral activities in untreated MDBK cells. These induced activities are abolished by anti-bovine rIFN-β antibodies, but not by anti-bovine rIFN-α or -γ antibodies. These findings demonstrate that in response to ethanol treatment, the induction of 2'-5'A synthetase mRNA and activity as well as the establishment of the antiviral state in MDBK cells are due to the production of IFN-β. Ethanol-induced IFN has a titer value of 15 IU/ml and its maximum secretion occurs at 6 h.

Taylor et al. (34) found that raising the temperature increases antiviral activity as a result of de novo synthesis of IFN-γ in certain human B-lymphoblastoid cell lines transformed by Epstein-Barr virus, but not in nontransformed cell lines. It is known that MDBK cells are susceptible to infection with BDV; however, the production of IFN caused by BDV is not due to an activation of the virus because BDV infection-free MDBK cells cultured in medium supplemented with basal medium supplement produced IFN-β. Furthermore, anti-bovine rIFN-β antibodies abolished the 2'-5'A synthetase and antiviral activities induced in response to ethanol stress. These observations led us to conclude that ethanol-induced IFN, as was shown for poly(I).poly(C)-induced IFN (35), cannot act from inside the cells, but has to be released into the medium to bind to its receptor (36, 37) and then be internalized (38, 39) to induce 2'-5'A synthetase activity and the antiviral state.

We postulate that in ethanol-treated cells (like in heat-shocked cells) allowed to recover under normal growth conditions, the expression of the 2'-5'A synthetase gene may constitute part of the rescue mechanism in response to stress by increasing the decay of stress protein mRNAs through the 2'-5'A synthetase endonuclease pathway. An unusual class of phosphorylated dinucleotides (such as, for example, Ap4A) termed alarmones has been shown to accumulate after heat or ethanol stress in bacteria (40) and mammalian cells (41). Ap4A may also be involved in the regulation of the termination of the stress response in oocytes (42). Since we observed the synthesis of stress proteins in MDBK cells after ethanol or heat (19) treatment, we suggest that the 2'-5'-oligonucleotides synthesized, either in ethanol-treatedMDBK cells through the production of IFN-β or in heat-treated cells through the production of heat shock-induced factor, could also function as signal of the end of the stress response.

**Acknowledgments**—We are grateful to Dr. Thang for helpful discussions and constant encouragement. We thank Dr. J. Chebath for providing us with human 2'-5'A synthetase cDNA and Dr. S. Martinod for the generous gift of bovine rIFN-α, -β, and -γ and anti-bovine rIFN-α and -γ antibodies. We also thank Drs. C. Sripati and J. Hershey for their help during preparation of this manuscript and C. Denolf for typing the manuscript.

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Ethanol Induces Interferon-β Production

Figure 1: Polyacrylamide gel electrophoretic analysis of [3H]methionine pulse-labeled MDRB cell proteins during ethanol treatment and recovery. MDRB cell proteins were pulse-labeled for 1 h periods with [3H]methionine and analyzed by electrophoresis on 10% (w/v) polyacrylamide gel (30,000 counts/sample) as described under "Experimental Procedures." Lane C: labeling of untreated cells in normal growth conditions during 1A, lane 1; 1/2A, lane 2; 5/6A, lane 3; 5/7A, lane 4; and 7.5/8A, lane 5. Lane D: labeling of ethanol-treated cells in ethanol-containing medium during 1A, lane 1; 1/2A, lane 2; and 5/6A, lane 3. Lane E: labeling of ethanol-treated cells in normal growth conditions during 1A, lane 1; 1/2A, lane 2; 5/6A, lane 3; 5/7A, lane 4; and 7.5/8A, lane 5. The arrows on the right indicate the molecular weight standards in kDa; phosphorylase B (97,000 daltons), bacitracin (61,000 daltons), ovalbumin (45,000 daltons), carbonic anhydrase (30,000 daltons). The arrow on the right points to the 30 kDa band.

Figure 2: Effect of ethanol on MDRB cell growth. MDRB cells were treated with 7.5% (v/v) ethanol in medium supplemented with serum; after 1 h, the medium was replaced with ethanol-free medium and cells were incubated at 37°C. At the times indicated, untreated (○) and ethanol-treated cells (●) were counted.

Figure 3: Kinetics of formation of 25 A synthetase activity in MDRB cells at 37°C after 1 h ethanol treatment. MDRB cells (1.5 x 10^6) were exponentially grown at 37°C with 7.5% (v/v) ethanol in a medium containing fetal bovine serum; after 1 h, ethanol was removed and cells were incubated at 37°C in 7.5% ethanol-free medium. At the indicated times, 25 A synthetase activity was determined in extracts of untreated (○) and ethanol-treated cells (●) as described under "Experimental Procedures."