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THE EFFECT OF HALOGENATED AMPHETAMINES ON PROTEIN SYNTHESIS IN NEWBORN RATS

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Abstract—The effects of *p*-chloroamphetamine and fenfluramine on protein synthesis in newborn rats was studied. Proteins from brain and limb muscles were analyzed by acrylamide SDS gel electrophoresis. Several major proteins detected by this procedure showed a reduced synthesis, induced by the drugs. The most noted effect was in a 34,000 dalton (34K)polypeptide that is more abundant in muscle than in brain. This polypeptide, as well as the adjacent 32,000 dalton (32K) polypeptide, is probably plasma membrane associated. However the synthesis of only one of these two polypeptides is affected by the halogenated amphetamines. Tropomyosin is similar in size to the 34K polypeptide, therefore a possible identity between these two proteins was investigated. A small difference in size, and a distinct pattern of the peptides resulting from these proteins, by partial digestion, excluded such identity. Additionally, an effect of a single dose of those drugs on the development of the newborn rats was noted. A dose of 10–20 mg/kg *p*-chloroamphetamine or fenfluramine cause a significant retardation in weight gain within three days after the treatment. Treated animals are unable to overcome this induced difference in weight even during the following four weeks. The possibility of similar effects in humans is discussed.

d-Amphetamine sulfate and related compounds such as *p*-chloroamphetamine (PCA) or fenfluramine (Fen) have a wide range of effects on the central nervous system. The response is exhibited in heightened locomotor activity, catecholamine release, hypo- or hyperthermia and appetite depression [1–4]. The halogenated derivatives also cause serotonine depletion [5, 6]. Recent studies have demonstrated the effect of amphetamines on the disaggregation of brain polysomes [7]. The use of cell-free systems revealed their action as inhibiting the initiation of protein synthesis [8]. Additionally, with the utilization of the wheat germ cell-free system, it was possible to show a selective effect on viral RNA translation [9].

Recently we have demonstrated that *d*-amphetamine sulfate and its halogenated derivatives, PCA and Fen, inhibit specifically the synthesis of certain proteins in cultured myotubes [10]. Since this selective effect was shown only in cell-free systems and tissue cultures, we undertook this study to determine whether such a selective effect exists *in vivo*. In order to label proteins of drug-treated animals to a high extent with a radioactive amino acid, we used newborn rats. The newborns have a small body weight and a high rate of protein synthesis. Thus it is possible to obtain in them proteins labeled to a high specific activity. The highly labeled proteins are essential for the detection of differences in the rate of synthesis. However, the disadvantage of newborn rats is their low sensitivity to the neurotoxic effects of the halogenated amphetamines. While a single dose of 7.5 mg/kg or more of PCA or Fen causes a long lasting serotonin depletion in adult rats, newborns recover within two weeks [11, 12]. Therefore, the effects of halogenated amphetamines exhibited in newborn rats are of a limited extent. Despite this limitation, the newborn rats demonstrate behavioural symptoms similar to adults immediately upon injection of the drugs.

In this study we show that Fen and PCA affect synthesis of some proteins in the limb muscle of newborn rats similar to the effect observed in cultured muscle cells. Brain proteins show a more limited effect. Since the most affected protein was a 34,000 polypeptide, we have examined a possible identity between actomyosin and this polypeptide, as suggested in a previous study [10]. Additionally, the long lasting effects of these drugs on weight gain in developing rats was studied.

MATERIALS AND METHODS

Newborn rats and mothers were Sprague–Dawley, Fischer and Sabra strains from the Weizmann Institute breeding center. Myotubes from chick chest muscle were prepared from 11–12 days embryos. *p*-Chloroamphetamine was from Sigma. Fenfluramine came from Avik, Israel. [^{35}S]-L-Methionine (750–1200 Ci/mM) was purchased from the Radiochemical Center, Amersham. *Staphylococcus aureus* protease was from Miles. Gradient acrylamide SDS gels were prepared according to Laemmli [13] with the modification described [10]. Autoradiography and the absorbancy scans of the autoradiograms were performed as described [10]. Actomyosin complex was isolated from rat muscle and chick cultured myotubes by the method of Carmon *et al.* [14]. Peptide mapping experiments were performed according to the method of Cleveland *et al.* [15].

RESULTS

Newborn rats (immediately after birth, up to 6 hr later) were injected through the tail with 0–20 mg/kg PCA or Fen. Two or three animals from one litter were injected with each of the selected drug concentrations. The animals were kept with the mother for an additional 12 hr. One hour before killing, each of the animals selected for the labeling experiment

was injected with 250–500 μCi of ^{35}S-methionine (5–8 μCi/μl). The brain and limb muscles were removed and homogenized with the aid of an 'Ultra Turrax' homogenizer in 0.5 ml phosphate buffered saline (PBS). 100 microlitre samples of the homogenate were immediately mixed with 2 vol. of 62 mM Tris–HCl, ph 6.8, containing 5% 2-mercaptoethanol 3% sodium lauryl-sulfate (SDS) and 0.01% bromophenol blue (sample buffer) and boiled for 10 min. The organs from each animal were treated separately. For sub-cellular fractionation, the homogenate was centrifuged at 1000 *g* (10 min) to remove unbroken cells and tissue pieces, then it was further centrifuged at 10,000 *g* (10 min) to remove nuclei and mitochondria. Finally the 10,000 *g* supernatant was centrifuged at 100,000 *g* (60 min) to recover the membranous and particulate fraction which was designated particulate fraction. The 100,000 *g* pellet was suspended in 150 μl sample buffer and the supernatant was mixed with 2 vol. of the same buffer and boiled. The incorporation of ^{35}S-L-methionine into these fractions was determined as hot trichloroacetic acid (TCA) insoluble radioactivity.

Protein quantitation was performed on samples without detergent by the differential absorbancy method measurement at 228.5 nm and 234.5 nm [16].

The specific activity of ^{35}S-L-methionine incorporated *in vivo* was 3–5 $\times$ 10^5 cpm/mg protein in brain extracts and 2–4 $\times$ 10^5 cpm/mg protein in muscle extracts. The specific activity of ^{35}S-methionine incorporation into proteins from drug treated animals was not reduced as compared to control animals.

Figure 1 shows an autoradiogram of total proteins solubilized from brain and limb muscle of newborn rats labeled for 1 hr with 250 μCi ^{35}S-methionine. The first slot in both groups presents the proteins from control animal, while the others are from animals treated with increasing concentration of Fen as indicated. The last slot presents the labeled proteins from cultured chick embryo myotubes.

The amount of radioactivity incorporated into certain polypeptides may vary among the various animals used for experimentation. However, such differences are difficult to attribute to drug effect, since they might be just an artifact of the varying amounts of radioactive amino acid injected. To overcome this difficulty, we have compared the ratio of radioactivity in the various polypeptides from the same animal.

To determine whether the synthesis of certain proteins was affected to a greater extent than others by the administration of Fen or PCA, the autoradiograms of the gels were scanned at 560 nm (Fig. 2). By this procedure, a graphic demonstration of the amount of radioactivity in each polypeptide band is obtained, assuming a linear relationship between the amount of radioactivity and the darkening of the photographic film. The most abundant proteins—actin, tubulin and myosin—may incorporate large amounts of ^{35}S-methionine and thus darken the photographic film to its full capacity. Therefore in such case the linear relationship between the amount of

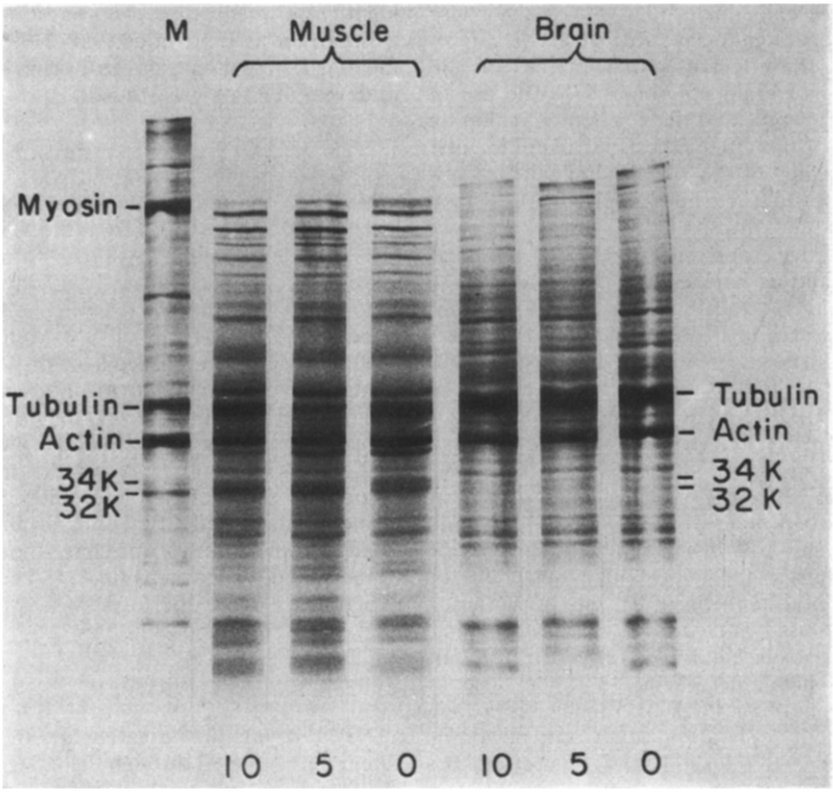

Fig. 1. An autoradiogram of newborn rats proteins labeled *in vivo* with ^{35}S-methionine. The first slot from left shows chick myotubes proteins followed by three samples of muscle extract and three from brain of Fen treated animals. The concentration of the drug in mg/kg is indicated under each run of the gel.

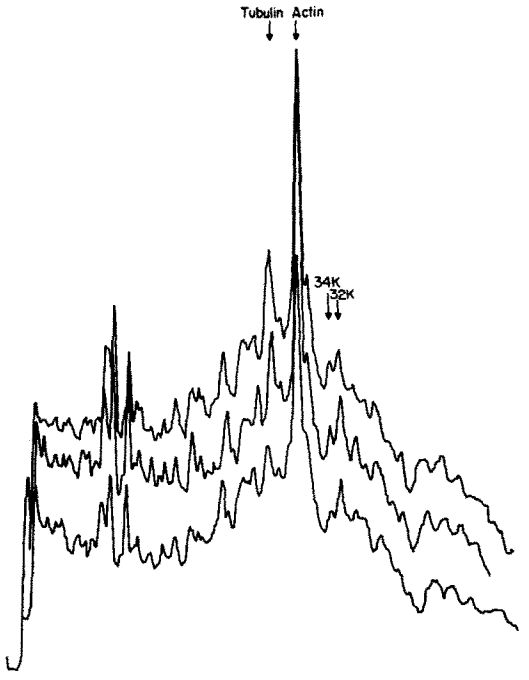

Fig. 2. A 560 nm absorbancy scan of autoradiograms of acrylamide gradient SDS gel, on which ^{35}S-methionine labeled proteins were separated by electrophoresis. The lines from top represent the radioactive amino acid incorporated into control animal limb muscle, treated with 5 mg/kg Fen and treated with 10 mg/kg Actin and tubulin serve as internal markers. The 34 and 32K polypeptides are indicated by arrows. Note the relative reduction in the 34K as compared to the 32K. Such a comparison is unaffected by total amount of radioactivity applied to each gel run.

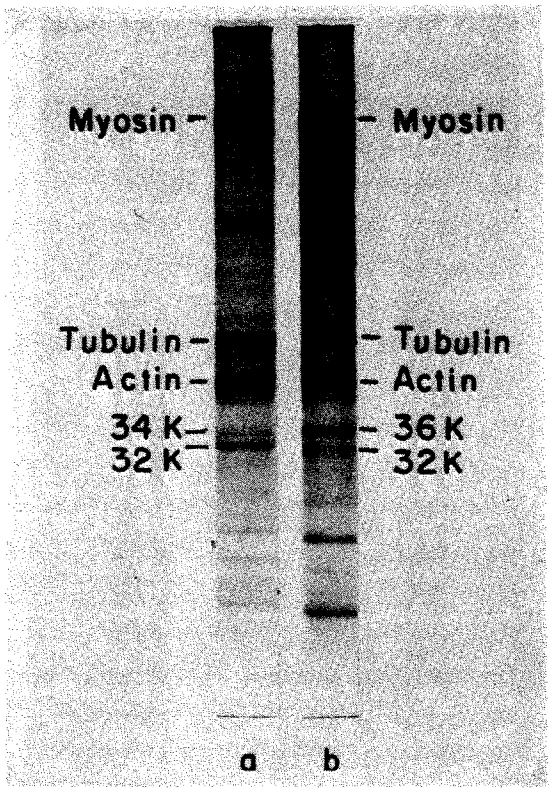

Fig. 3. This figure shows an autoradiogram of ^{35}S-methionine labeled proteins from chick embryo cultured myotubes separated by gel electrophoresis. Total homogenate (a). The proteins of the actomyosin complex (b). The difference in mobility between the 34K polypeptide from the total homogenate and the enriched band of 'tropomyosin' is apparent, and is estimated as 1500 dalton. The band of the 34K was completely missing from the actomyosin complex. CarMV RNA *in vitro* translated products of 77,000, 38,000 and 30,000 with myosin heavy chain 200,000, tubulin 55,000 and actin 44,000 served as molecular weight markers.

radioactivity and dark grains in the photographic emulsion will not exist any more. Since we compare proteins other than those most abundant, we can consider the smaller peaks as in the linear range of response. From both Figs. 1 and 2 it is apparent that the synthesis of the 34,000 dalton (34K) polypeptide extracted from newborn rat limb muscle is affected more than any other major protein. The adjacent 32,000 dalton (32K) polypeptide which is least affected serves as reference to this decline. In the brain extract such an effect can be noted, but it is less obvious (Fig. 1). Since the quantity of radioactivity in the 32 and 34 K bands in the brain extract was small relative to the other major proteins and to the amount found in muscle extract, the scanning of such an autoradiogram at 560 nm did not clarify the pattern and thus those results are not shown. An additional advantage to the use of the total homogenate is to avoid the possibility of selective loss of certain fractions from the tissue homogenate and creation of artifacts. However since the 34K polypeptide is probably plasma membrane associated, purified fractions of this membranous structure may help to establish the biological role of this protein. Since the 34K was the most susceptible polypeptide in limb muscles as well as in myotube cultures, we used tissue cultures to examine a possible identity between this polypeptide and tropomyosin, a possibility that was suggested in a previous work [10].

For this purpose, four day old cultures were labeled with ^{35}S-methionine 8 μCi/ml for 4 hr. A sample of the labeled cells was disrupted without any subcellular fractionation, while the bulk of the cells were used for separation of the actomyosin complex by the method of Carmon *et al.* [14]. The myotubes total homogenate and the actomyosin complex were further analysed by SDS acrylamide gel electrophoresis. The results of such a fractionation are demonstrated in Fig. 3. The 34K polypeptide is missing from the actomyosin complex, while the calculated mol. wt of the enriched band, assumed to be the tropomyosin, is 35,500 daltons. The 1500 dalton difference in mol. wt between those two bands is apparent from the autoradiogram presented; this difference was reproduced in several experiments. Furthermore, since the difference in size is small, another experimental approach was used for comparing these two proteins. The corresponding bands, as determined by stained gels and autoradiography, were excised from the dried gel and partially digested by *S. aureus* protease according to Cleveland *et al.* [15]. The resulting peptides were resolved by gel

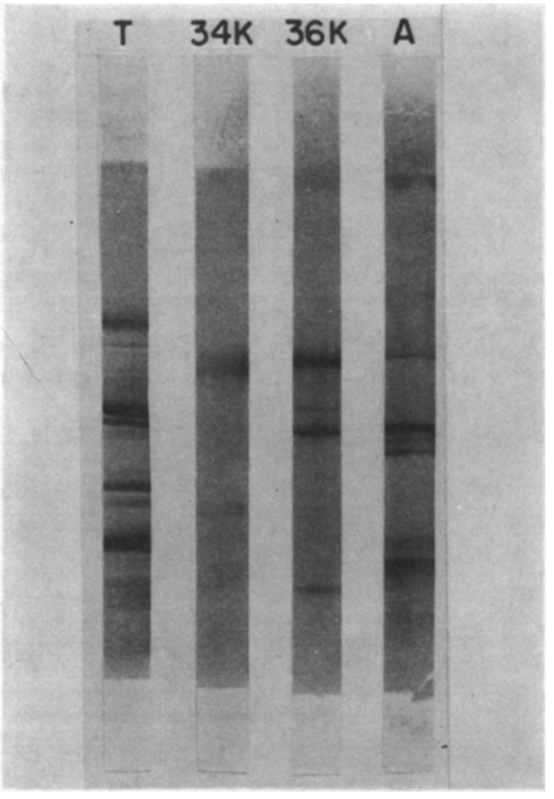

Fig. 4. Peptide mapping of the 34K polypeptide and 'tropomyosin' 36K. The corresponding bands with actin and tubulin as markers were partially digested by *S. aureus* protease and separated by electrophoresis on 15–20% acrylamide gradient SDS gel. The autoradiogram of the ^{35}S-methionine resulting from A—actin, 34 K polypeptide, 36 K polypeptide and T—tubulin is shown.

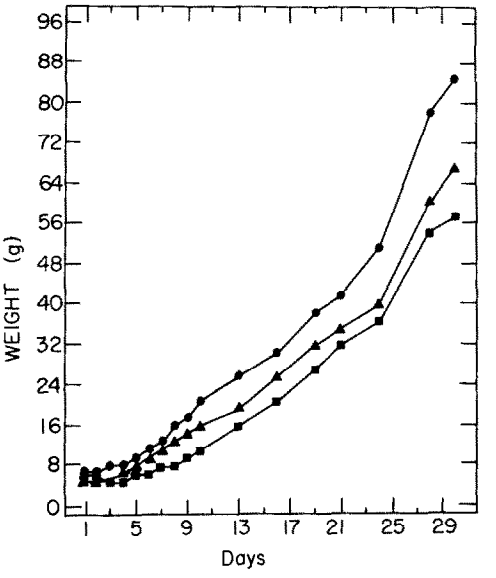

Fig. 5. This figure demonstrates the long-lasting effect of Fen on weight gain by newborn rats. Newborn rats were given a single injection of Fen—2.5, 5, 10 and 20 mg/kg. The control animals were injected with equal volume of phosphate buffered saline. ●——● control; ▲——▲ 10 mg/kg; ■——■ 20 mg/kg. Note the equal initial weight.

electrophoresis and compared to each other (Fig. 4). Partial proteolytic digest yielded a different peptide profile for these two proteins, supporting the observation based on size determination that these are two different and distinct proteins.

Since only a few animals from each litter were used for the labeling of their proteins with ^{35}S-methionine, the rest of each litter was kept with the mother for an additional four weeks to trace the long lasting effects of the drugs. During this period the animals were weighed daily for the first 10 days and then once every three days. The administration of high doses of PCA or Fen caused the treated newborn rats to gain significantly less weight than the controls or those treated with low doses of the drugs (Fig. 5). The difference in weight can be noticed within 48 hr after injection of the drug. Moreover the newborns which received 20 mg/kg Fen showed a significantly smaller weight even four weeks after the single injection (Fig. 5), although full recovery in serotonin level was reported by that time [12]. The results presented in Fig. 5 are based on one litter; additionally, three other experiments showed precisely the same weight gain pattern. To determine the significance of the effect of drugs on retardation of weight gain, a statistical analysis of variance was performed. This analysis showed the differences in weight to be significant with $P \leq 0.0001$. A single injection of 2.5 or 5.0 mg/kg of the drug did not result in detectable differences in weight; however 10 or 20 mg/kg had an observable effect within the first week following injection from which the animals were unable to recover during the following three weeks.

Special attention was given to the initial size of the animals compared, since large differences in the birth weight obscure the drugs' effect during later periods.

DISCUSSION

p-Chloroamphetamine and fenfluramine are known as appetite depressants to cause long lasting neurotoxic effects and to disaggregate polysomes in rat brain [7, 11, 12]. In addition, these drugs were shown recently to cause selective inhibition of protein synthesis in cultured muscle cells [10]. Therefore it was of interest to learn whether this selective inhibition occurs only in cultured cells or if it is a general phenomenon occuring *in vivo*. However, a major difficulty encountered in such an *in vivo* study is the small amount of radioactive amino acid incorporated into the proteins of the organs of treated animals. Moreover, to detect minute differences caused by a drug-reduced rate of protein synthesis, a high specific-activity radioactive label is essential. ^{35}S-Methionine provides a radioactive amino acid of very high specific activity and additionally it has limited pools in several tissues, including brain [17]. Therefore this radioactive amino acid can yield highly labeled proteins *in vivo*; in fully grown rats (even in young adults) the rate of protein synthesis is low as compared to embryonic tissues or newborns. Additionally, the size of such animals will result in

a dilution factor of 20–50 fold greater than in newborns. Thus to achieve high specific labeling of proteins with a radioactive amino acid, very large quantities would be required, which makes such experimentation impractical. To overcome these difficulties, newborn rats were utilized, although PCA or Fen cause only limited neurotoxic effects in them [12]. The high specific labeling of proteins obtained by a short pulse of ^{35}S-methionine given to these animals enabled us to detect differences in protein synthesis *in vivo*.

From the data on cultured cells it was anticipated that cellular proteins such as actin and tubulin would not be affected by the halogenated amphetamines. However, since the method employed for resolving cellular proteins in an unfractionated homogenate is not sensitive enough to trace proteins that comprise less than 0.1% of the tissue proteins, only differences in major proteins could be detected. *A priori*, this means that differences in rates of enzymes synthesized will not be detected by this procedure.

Nevertheless, the procedure of autoradiography of SDS acrylamide gels was sufficiently sensitive to show a reduced rate of synthesis of a membrane associated protein, the 34K polypeptide (S. Paglin, Ph.D. thesis and personal communication). The content of this polypeptide in brain extract is probably smaller than in muscle, therefore the results obtained by electrophoretic separation of brain extract did not show the same clear reduction as was observed in the limb muscle extracts. Reduced radioactivity in the 34K polypeptide as compared to adjacent 32K polypeptide was noticed in some of the brain extracts. Since the relative quantity of these two polypeptides is smaller in brain, their appearance on the autoradiogram was sometimes blurred. Attempts to enrich this fraction by centrifugation and removal of other subcellular components did not improve resolution. Therefore the fractionation approach was not pursued. A possibility exists that the drug treatment may alter membranous structures in the cells and thus fractionation may yield different quantities of a particular structure in drug treated animals. To avoid such a possible artifact, total homogenates were resolved. The most sensitive polypeptide, the 34K, is similar in size to tropomyosin; thus we have examined a possible identity. However, size estimation by gel electrophoresis and peptide mapping indicated that these are two different and distinct proteins. One of the above-mentioned drugs (Fen) is widely used as an appetite supressant [4, 18] and commonly given to hyperactive children. Therefore it was of interest to examine whether Fen affects protein synthesis *in vivo*. The selective effect on the synthesis of some major proteins may just be an indication to similar effects on some enzymes, thus further studies will be required to elucidate this point. The long-lasting retardation in weight induced in animals treated once with a high dose of the drug may as well result from repeated small doses [12]. Thus this observation indicates possible damage caused by the use of Fen and indicates the necessity of a new evaluation of its broad use.

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