tissues from the group made anemic by bleeding showed *increased* porphyrinogenesis as compared with the controls. The same results were obtained in dogs similarly treated but administered added iron. It is suggested that the decrease of porphyrin biosynthesis observed in the tissues from the phenylhydrazine-treated dogs may result from an *in vivo* "feedback" inhibition by coordinate repression by heme or possibly a related metabolite. The increased porphyrinogenesis observed in the tissues of the dogs made anemic by bleeding may be related, either directly or indirectly, to erythropoietin or to some other hemopoietic factor.

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Lack of Transfer of Morphine Tolerance by Administration of Rat Cerebral Homogenates* (32900)

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It has been reported by Ungar and Cohen (1) that tolerance to morphine can be transferred to mice by injection of homogenates of the brains of morphine-tolerant rats and dogs. They tentatively characterized the transfer factor as a small protein or peptide. Their results could prove to be very significant in explaining the mechanism of tolerance to narcotic analgesics since protein synthesis has been implicated in the development of morphine tolerance (2–4). However, recently Tirri (5) was unable to demonstrate the transfer of morphine tolerance from tolerant mice to normal mice.

The present report represents a study of the replicability of the transfer of morphine tolerance from tolerant rats to normal mice. If the development of tolerance does involve the synthesis of a "tolerance factor" which can be transferred, its transfer should have replicability and generality so this phenomenon can be more widely studied. This work was undertaken with this in mind.

Materials and Methods. The procedures followed were similar to those used by Ungar and Cohen (1); the only major modification was the use of heat, instead of pressure, as the noxious stimulus in the determination of analgesia. Male Holtzman rats weighing between 160 and 200 gm were injected subcutaneously with morphine sulfate t.i.d. at 4-hour intervals with doses increasing progressively from 10 mg/kg per injection on the first and second days to 100 mg/kg per injection on days 13 and 14. On day 15 one injection of 100 mg/kg was given 8 hours before sacrifice. Control rats received injections of 0.9% saline according to the same schedule.

All rats were sacrificed by decapitation. Cerebral hemispheres were removed and weighed rapidly, frozen on dry ice, and stored at −80°C until used. On the day of the experiment the cerebral hemispheres in each group were pooled and homogenized in 1.5

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volumes of 0.9% saline using 10 strokes with a Pyrex tube–Teflon pestle tissue grinder (Thomas).

The recipients of the homogenates were adult male Swiss albino mice weighing between 25 and 35 gm. The mice were injected intraperitoneally with either 0.9% saline (0.5 ml/30 gm of mouse) or homogenate (0.5–0.6 ml/30 gm of mouse representing 200–240 mg of rat brain) 3 hours before a subcutaneous injection of a test dose of morphine sulfate.

Analgesia was determined by the hot plate method of Eddy (6). The room temperature was kept constant between 25 and 27°C. A metal container was placed in a constant temperature bath held at 30°C and each subgroup of 10–12 mice was allowed to equilibrate in this container for 10 min before testing.

Three control reaction times were determined for each mouse. A mouse with any one of its reaction times more than 2 standard deviations from the mean of the group was eliminated from the study. The morphine test dose was considered to be exerting an analgesic effect if at 60 min after injection the reaction time of the mouse was longer than its own mean control time plus 2 standard deviations of the group.

In one experiment a supernatant fraction was used since the transfer factor was tentatively characterized as a small protein or peptide (1). Homogenates were prepared as above and centrifuged at 2000*g* for 20 min at 0°. Seventy percent perchloric acid was added to the decanted supernate to attain a final concentration of 3% perchloric acid. The precipitate was removed by centrifugation and the supernate was neutralized by addition of solid K_2CO_3. After the removal of the precipitated $KCLO_4$ by centrifugation, the resulting supernatant fraction was tested in mice as described above (0.6 ml/30 gm of mouse representing 380 mg of rat brain).

Dose–response curves were tested for parallelism and ED_{50} values were calculated and compared using the method of Litchfield and Wilcoxon (7). Statistical evaluation of the changes in reaction time was performed using the Student *t* test.

Results. In the first experiment the mean control reaction time and standard deviation for the group which subsequently received saline was 7.8 ± 3.2 sec. These were obtained from 3 observations on each of 48 mice in this group. The control values for the groups receiving the cerebral homogenates from the saline-treated and morphine-treated rats were

TABLE I. Test of the Transfer of Tolerance to the Analgesic Effect of Morphine.

Pretreatment	Morphine test dose (mg/kg)	No. of mice showing analgesia/ no. of mice tested	% showing analgesia	Slope[a] function (95% C.L.)	ED_{50}[a] (95% C.L.) (mg/kg)
Saline[b]	2	1/12	8		
	4	3/12	25	2.03	6.0
	8	8/12	67	(1.35–3.04)	(4.0– 8.9)
	16	11/12	92		
Cerebral homogenate	4	3/10	30		
from saline-	8	6/12	50	2.49	7.3
treated rats[c]	16	10/12	83	(1.24–4.98)	(4.7–11.3)
Cerebral homogenate	4	1/11	9		
from morphine-	8	7/12	58	2.72	10.8
treated rats[c]	16	6/12	50	(1.13–6.53)	(6.8–17.1)

[a] Calculated according to the method of Litchfield and Wilcoxon (7). There was no significant difference between any of the groups in either the slope function or ED_{50}.

[b] Saline was injected in a volume equal to the volume of homogenate injected (0.5 ml/30 gm of mouse.

[c] The homogenates were prepared as described in "Materials and Methods" and were injected intraperitoneally 3 hours before the injection of the test dose of morphine sulfate. The amount injected was 0.5 ml/30 gm of mouse representing 200 mg of rat brain.

7.6 ± 2.8 sec and 7.8 ± 2.9 sec, respectively, showing that the mice in the 3 groups were likely from the same population.

The results of the first experiment are presented in Table I. The 3 main groups of mice, representing animals receiving the 3 different pretreatments, were divided into 3 or 4 subgroups of 12 mice each. Each subgroup was treated as a unit. It was assigned one test dose of morphine and all the mice in each subgroup were tested together. Although the dose–response curves obtained from mice pretreated with cerebral homogenates from saline-treated or morphine-treated rats were not significantly different from the dose–response curve of the saline-pretreated mice, there was a suggestion of transfer of tolerance by the cerebral homogenate from the morphine-treated rats when a test dose of 16 mg/kg was used. However, there was no indication of tolerance to the 8 mg/kg test dose in this same group. To resolve this conundrum both of these dose levels were repeated.

Several modifications were made in all subsequent experiments. In each experiment the mice were divided into 3 groups of 10–12 mice each. After taking 3 control times on each mouse it was randomly assigned a pretreatment but left in its predetermined group. The observer was not aware of either the mean control time or the pretreatment of any given mouse. The mean control time and standard deviation for the group tested with 8 mg/kg morphine was 7.2 ± 2.8 sec. Reaction times were determined at hourly intervals after the morphine injection. The mean changes in reaction times with standard errors are shown in Fig. 1. None of the values is significantly different from that of the saline control group.

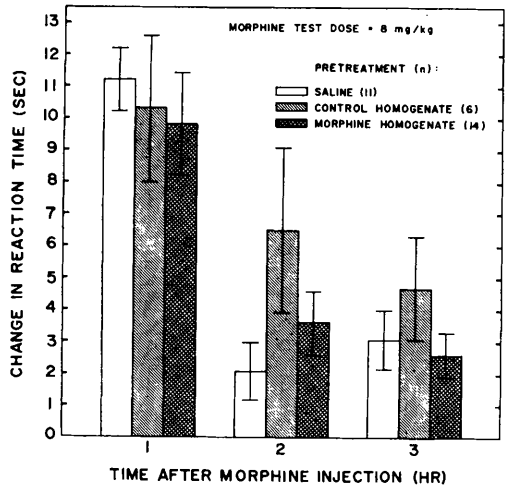

FIG. 1. Increase in reaction time (mean ± SE) from control reaction time due to an 8 mg/kg test dose of morphine sulfate injected 3 hours after pretreatment. The pretreatment was either saline (0.5 ml/30 gm of mouse) or cerebral homogenate (0.5 ml/30 gm of mouse representing 240 mg of rat cerebral tissue) from either saline-treated or morphine-treated rats. The number of mice in each group is indicated by (*n*).

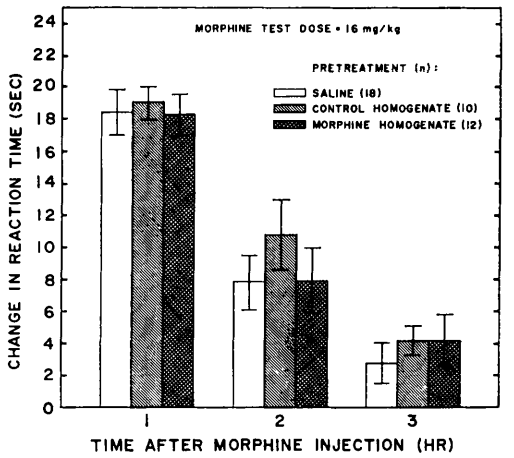

FIG. 2. Increase in reaction time (mean ± SE) from control reaction time due to a 16 mg/kg test dose of morphine sulfate injected 3 hours after pretreatment. The pretreatment was either saline (0.5 ml/30 gm of mouse) or cerebral homogenate (0.5 ml/30 gm of mouse representing 240 mg of rat cerebral tissue) from either saline-treated or morphine-treated rats. The number of mice in each group is indicated by (*n*).

The mean control time and standard deviation for the group tested at 16 mg/kg was 7.6 ± 3.0 sec. The results of this experiment are presented in Fig. 2. Again, there were no significant differences in the mean change in reaction time at any of the observation times.

The results obtained using the supernatant fraction with the morphine test dose at 16 mg/kg are shown in Fig. 3. The above modifications of random testing were also incorporated into this experiment. The mean control time and standard deviation for this group was 7.8 ± 3.2 sec. The figure illustrates

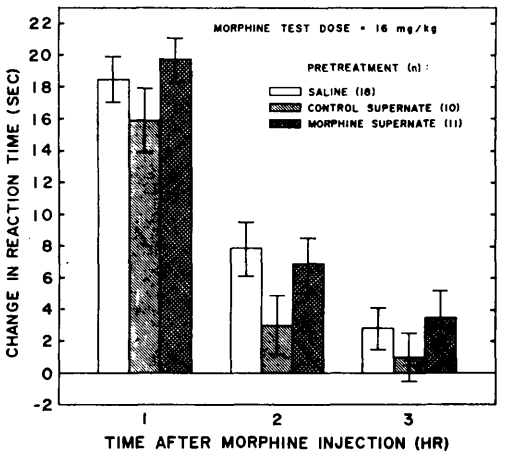

FIG. 3. Increase in reaction time (mean ± SE) from control reaction time due to a 16 mg/kg test dose of morphine sulfate injected 3 hours after pretreatment. The pretreatment was either saline (0.5 ml/30 gm of mouse) or a supernatant fraction (0.6 ml/30 gm of mouse representing 380 mg of rat cerebral tissue) prepared from cerebral homogenates from either saline-treated or morphine-treated rats. The number of mice in each group is indicated by (*n*).

the mean changes in reaction times at hourly intervals after the injection of the test dose of morphine and again there were no significant differences.

Discussion. Neither the dose–response curves nor the mean changes in reaction times of the animals pretreated with homogenates from saline-treated or morphine-treated rats were significantly different from those of animals pretreated with saline. After the first experiment the mean change in reaction time was recorded at hourly intervals because it was felt that this would be a more sensitive index of analgesia rather than the quantal results recorded in Table I. It can be seen in Figs. 1, 2 and 3 that neither the peak change in reaction time nor the duration of action of the test dose was decreased by either the cerebral homogenate or supernatant fraction from morphine-tolerant rats.

The results obtained in this study do not confirm the results of Ungar and Cohen (1). There are at least two possible explanations for the negative results obtained concerning the transfer of morphine tolerance. In the transfer of induced tolerance there may be subtle but very important differences in experimental procedures which may eventually account for the differences in results. Examples of factors which may be critical are the duration of the interval from the decapitation of the rats to the freezing of the brains, the temperature at which they are stored, and the duration of storage before use. Additionally, we used the hot plate method to determine analgesia while Ungar and Cohen (1) used a pressure method. In this regard, however, Tirri (5) was unable to demonstrate the transfer of morphine tolerance with either method.

The second explanation involves the experimental design used in this study. As Tirri (5) concluded after obtaining negative results in the transfer of morphine and promazine tolerance, there may be certain experimental precautions which are essential for studies of this nature. The following precautions were included in this study and should be heeded in all transfer studies. Firstly, there should be complete randomization so that mice of any given pretreatment are not tested as a group. Secondly, the observer should lack the knowledge of the mean control time and of the pretreatment of any individual mouse.

If a transferable "tolerance factor" is indeed a fact, one should be able to demonstrate a transfer of tolerance in spite of the random testing described above. Using the random testing procedure, however, we were unable to find any evidence for the presence of a transferable "tolerance factor" in the cerebral homogenate of morphine-tolerant rats. At first glance, the above findings may appear to contradict the view that tolerance is related to some type of protein synthesis. This need not be the case. It may merely mean that if a "tolerance factor" is synthesized during the chronic treatment with narcotics, the transferability of this factor is equivocal.

Summary. Transfer of tolerance to the analgesic effects of morphine to mice by injection of cerebral homogenates from morphine-tolerant rats was studied. After such pretreatment the mice were injected with a test dose of morphine and then tested for analgesia using the hot plate method. Nei-

ther the dose–response curve nor the mean increase in reaction time was significantly altered by pretreatment with the cerebral homogenate from morphine-tolerant rats. The possible reasons for the lack of confirmation of results obtained by other workers were discussed.

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Carcinogenicity of Long-Term Feeding of Cycad Husk to Rats* (32901)

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Cycads are used as food on Guam and other areas in the tropics and subtropics (1, 2). The cycad, *Cycas circinalis*, is native to Guam. Guamanians wash and grind the kernels of the seeds of the *C. circinalis* to produce a starchy flour resembling wheat flour in appearance and use (3). This cycad flour or processed kernel obtained from local markets on Guam was noncarcinogenic when fed to rats whereas the unprocessed (other than vacuum drying and grinding) kernels were toxic and carcinogenic (3–7).

The fleshy covering or husk of the kernel is used to relieve thirst or when dried is eaten like sweets (1). The use of the husk has special public health implication in that it is not processed. Children reportedly eat the husk since it is sweet and readily available. The acute toxicities of the husk have been demonstrated in rats.[1] These include anemic appearance, hemoconcentration, ascites, weight loss, and early mortality. The present experiments were designed to utilize cycad husk removed from cycad kernels obtained from Guam and the objective was to determine whether long-term ingestion of low levels of cycad husks would produce tumors.

Methods. A total of 120 Sprague-Dawley rats were used in 3 trials. Trials 1 and 2: 10 rats of each sex were fed a diet containing 2.0% or 1.0% husk[2] respectively. Five control rats of each sex were included. Trial 3: 10 rats of each sex were fed diets containing 0, 0.5, or 1.0% husk. Control rats were fed a basal diet[3] without husk.

All rats were housed in individual suspended wire cages and were fed the husk diets or the basal diet and water *ad libitum* starting at 26 days of age. Body weights were recorded weekly. A complete necropsy was performed on all rats which died or when they were killed. The surviving rats in trial 3 were killed and necropsied at the end of 253 days of feeding when the experiment was terminated. Gross lesions were recorded at the time of necropsy. Eighteen different tissues were

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[1] Yang, M. G. and Mickelsen, O., 1967, manuscript submitted for publication.

[2] We thank Drs. L. T. Kurland and M. G. Whiting of the Institute of Neurological Diseases and Blindness, Bethesda, Md. for the generous supply of processed *Cycas circinalis* kernals and husks.

[3] Composition of basal diet has been described in footnote 6 of ref. (3).