Nonspecific Behavioral Effects of Substances from Mammalian Brain

Frank, Stein, and Rosen (*1*, *2*), have reported that mice injected intraperitoneally with either brain or liver homogenate from donors that had been shocked in a black box after escaping an intense light in a white box, or stressed by being tumbled in a jar, had longer latencies to enter the black box than recipients injected with homogenate from control donors allowed to escape intense light but not shocked. These data were interpreted as indicating that the interanimal transfer phenomenon reported by us and others may involve transfer of a nonspecific stress-associated factor unrelated to memory.

Although we find ourselves in sympathy with the suggestion made by Frank *et al.*, that some of the observed results from the transfer-type experiments, particularly in aversive training situations, may be attributable to emotion factors induced or released in donor animals during their training, we are not persuaded that the evidence in their report provides reliable support for this hypothesis, or is a relevant basis for criticism of other work with which it is compared.

First, Frank *et al.* claim that "successes" reported by others have generally involved the use of passive avoidance, while "failures" have frequently involved the use of positive reinforcement situations. To the best of our knowledge, there have been only two studies reported in which an attempt was made to transfer a true passive avoidance task (*3*). Both attempts were unsuccessful, although Ungar and others (*4*) have reported considerable success with the use of an avoidance box somewhat similar to that used by Frank *et al.*, but with a quite different donor training paradigm and recipient testing schedule. Frank *et al.* cite no actual experiments later than 1967: of the three early "successes" cited to make their point, none involved the use of a passive avoidance paradigm; rather, two used positive reinforcement (*5*) which Frank *et al.* incorrectly state generally leads to failure, and one employed habituation (*6*). In point of fact, better than half of the successful studies described have employed positive reinforcement, and "failures" have been reported at least as frequently with avoidance paradigms as with positive reinforcement paradigms (*7*).

Second, Frank *et al.* state that "in the avoidance task, an increase in latency of recipients when compared with those of donors is taken as evidence of . . . memory transfer." We disagree. The critical comparison is almost always between recipients injected with brain material from untrained or control group donors and recipients injected with material from trained donors. Furthermore, active avoidance studies (*8*), in which a *decrease* in latency is taken as evidence of transfer, are ignored by Frank *et al.*

Third, Frank *et al.* state that recipients were tested 6 hours after injection of organ homogenate, by which time observation of animals in their home cages revealed "unequivocal" recuperation from incoordination and lethargy. Others, using objective measures of recovery, have reported that intraperitoneal injection of brain homogenate disrupts and depresses behavior for at least 24 hours (*9*). Frank *et al.* state that "increased latencies were observed for all recipient groups," compared with donors. This suggests failure of recuperation (since only two of the donor conditions were designed to involve stress or "stress substance") and thus clouds the interpretation of their main effects.

Fourth, if one considers the actual events involved in the "nonshocked" control condition, an interesting reconceptualization of their design is possible. In the "nonshocked" donor condition, intense light, an aversive stimulus for albino mice, was paired with the white compartment, while the black compartment was associated with relief from the light stimulus. If this association did indeed "transfer" in brain homogenate, what sort of latencies might one expect in recipients from this condition? Plainly, in the context of the "memory transfer" hypothesis which Frank *et al.* were testing, in these recipients the expected result would, if anything, be one of shorter latencies. This is in fact what they report.

Possibly the most interesting difference between the Frank *et al.* study and the successful "memory transfer" experiments of others is that Frank *et al.* administered but one brief training trial to their donors. No one else has ever described a positive transfer effect using one-trial learning paradigms to train donors; this problem has been discussed (*10*). With intraperitoneal injection, almost all investigators working on the transfer problem have reported that 1.5 to 2.0 brain equivalents of supernatant or extract (since 1967, whole brain homogenates have rarely been used) must be injected for an effect to be obtained. For both these reasons, even the nonspecific effect reported by Frank *et al.* in their one-trial situation is quite surprising and of considerable interest.

Their effect becomes less interesting, however, when their data are inspected closely. Table 1 shows, as reported by Frank *et al.*, the percentages of animals in each group with latencies greater than 20 seconds, with the complement obtained by subtraction; also shown in Table 1 are the actual number of animals corresponding to these percentages (*11*). These two halves of Table 1 allow somewhat different views of the magnitude of the comparisons reported by Frank *et al.* to be significant. For the two "significant" χ^2 values reported in experiment 1, neither is significant when calculated correctly from frequencies instead of percentages (*12*), with the continuity correction for χ^2 with one degree of freedom (*13*). Ac-

Table 1. Frequencies calculated from percentages reported by Frank *et al.* (*1*), for the six groups in each of their experiments. Dichotomization within each group is at 20-second latency to enter black compartment. S, shocked donors; NS, nonshocked donors; Str, stressed donors. Latency greater than 20 seconds, >; latency less than 20 seconds, <.

Recipient of:	Reported percentages (*1*)			Calculated frequencies*		
	S	NS	Str	S	NS	Str
			Experiment 1			
Brain >	25	15	33	5	3	7
<	75	85	67	15	17	13
Liver >	20	20	25	4	4	5
<	80	80	75	16	16	15
			Experiment 2			
Brain >	45	10	35	9	2	7
<	55	90	65	11	18	13
Liver >	50	30	50	10	6	10
<	50	70	50	10	14	10

* Our calculations.

Table 2. Comparisons reported significant with published values of χ^2 from Frank *et al.* (*1*); actual values of χ^2 also are shown. S, shocked donors; NS, nonshocked donors; Str, stresed donors. For example, *Brain: S versus NS* indicates comparison between recipients of brain homogenate from shocked and nonshocked donors.

Comparison		Chi-square values	
		Frank *et al.*	Actual*
		Experiment 1	
Brain:	S versus NS	9.6 †	0.16
Brain:	Str versus NS	7.9 †	0.12
		Experiment 2	
Brain:	S versus NS	26.3‡	4.51 §
Brain:	Str versus NS	16.5 ‡	2.29
Liver:	S versus NS	7.5 †	0.94
Liver:	Str versus NS	7.5 †	0.94

* Our calculation. † $P < .01$. ‡ $P < .001$. § $P < .05$.

tually, in view of the small expected frequencies in these tables (the expected frequency is less than five in two cells of each table), the "Fisher-Yates" test (*14*) is more appropriate and defensible. This test also fails to reveal significance in these two tables, by a considerable margin. Of the four "significant" χ^2 values presented by Frank *et al.* to support their conclusions in experiment 2, only one is still significant when calculated correctly (Table 2) or when the 2 by 2 table in question is tested by the Fisher-Yates method. This significant comparison is between recipients of brain material from "shocked" and "nonshocked" donors, the latter being identified in the abstract of their report as a "control."

Thus, of all the significant χ^2 values reported for both experiments, only one is actually significant—and this comparison is one which, in the context provided by Frank, Stein, and Rosen, would be taken as evidence for "memory transfer." However, considering the nature of this "control" condition, discussed above, and considering our other reservations (*15*) concerning these experiments, we are extremely cautious in accepting the evidence this comparison offers (*16*). Needless to say, were we persuaded that the "control" condition was really a control, we would consider the demonstration of memory transfer by Frank *et al.* more convincing.

In view of the foregoing considerations, it seems most inappropriate that Frank *et al.* attempt to relate their work to the interanimal transfer experiments. That both specific and nonspecific transfer effects exist has never been doubted by experimenters in the field, but adequate experimental design permits these effects to be disentangled. In their introductory discussion, Frank *et al.* note that their procedure was employed particularly to show nonspecific phenomena that "may have only a marginal effect" on measures typically used in transfer experiments. We are inclined to agree that their procedure does have this characteristic.

The transfer experiments are still controversial, primarily because those of us working in the field have not as yet been able to specify exactly all the factors affecting the phenomenon. Clearly, we share the interest of Frank *et al.* in determining additional factors that may confound the variables of interest in this type of research. To be of maximum value, such investigations should display care in experimental design and statistical analysis, caution in interpretation and generalization, and familiarity with the experimental work already described.

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References and Notes

1. B. Frank, D. Stein, J. Rosen, *Science* **169**, 399 (1970). All subsequent references to "Frank *et al.*" are to this reference. In a subsequent statement (*2*), Frank *et al.* acknowledge an error in the analysis of their data, but affirm that no consequent change is required in their original interpretation of the results.
2. B. Frank, D. Stein, J. Rosen, *Science* **177**, 638 (1972).
3. G. Ungar, personal communication; A. Cherkin, *J. Biol. Psychol.* **12**, 83 (1970).
4. G. Ungar, Ed., *Molecular Mechanisms in Memory and Learning* (Plenum Press, New York, 1970).
5. E. J. Fjerdingstad, Th. Nissen, H. Røigaard-Petersen, *Scand. J. Psychol.* **6**, 1 (1965); F. Rosenblatt, J. Farrow, W. Herblin, *Nature*, **209**, 46 (1966).
6. G. Ungar and C. Oceguero-Navarro, *ibid.* **207**, 301 (1965).
7. J. A. Dyal, in *Chemical Transfer of Learned Information*, E. J. Fjerdingstad, Ed. (North-Holland, Amsterdam, 1971), pp. 219–263.
8. An annotated bibliography of "memory transfer" experiments has appeared in M. Schulter, *J. Biol. Psychol.* **9** (2), 52 (1967); **10** (1), 80 (1968); **10** (2), 54 (1968); **11** (1), 56 (1969); **11** (2), 22 (1969).
9. M. L. Cheal, *ibid.* **12** (2), 21 (1970).
10. The apparent failure of one-trial learning to transfer has been discussed at several recent conferences (including at least two sponsored by AAAS in 1967 and 1970) by J. V. McConnell and others; see A. M. Golub, in *Methods in Neurochemistry*, R. Fried, Ed. (Dekker, New York, in press).
11. In calculating frequencies we assume that there were 20 animals in each recipient condition although it is possible to conclude that somewhat fewer animals were used in some cases.
12. The "statistical and typographical" error to which Frank *et al.* refer in their correction statement (*2*) was apparently the calculation of χ^2 values from percentages. By using percentages instead of frequencies all but one of the values they report in their paper can in fact be obtained. The reader may of course check for himself any of the χ^2 values reported in our comment or their paper.
13. For a convenient derivation of the continuity correction, see D. R. Cox, *Biometrika* **57**, 217 (1970).
14. R. E. Clark, *Critical Values in 2 × 2 Tables* (Department of Sociology, Pennsylvania State Univ. Press, University Park, 1969).
15. In addition to the problems enumerated in the body of our comment, we note the following. In experiment 1, Frank *et al.* use a one-tailed Mann-Whitney U-test. The comparisons for which the one-tailed test is employed seem to have been decided upon "as behavioral testing proceeded." The use of a one-tailed test is suspect in this situation. If one applies the conventional two-tailed criterion to the results of experiment 1, neither of the "liver recipients" differences is significant. In a footnote, the authors justify the use of a one-tailed test in both experiments "since we predicted recipient scores to be the same as, or longer than, donor latencies" (*1*, p. 402). What this has to do with the situation at issue is elusive, since they use the one-tailed values to test differences between recipient groups. We have considered the possibility that Frank *et al.* were really testing to see whether recipients had longer latencies than their respective donors, even though this is not stated. If they did this, it raises serious questions about the use of the Mann-Whitney test for independent samples in experiment 1, and invalidates its use in experiment 2. However, Frank *et al.* explicitly summarize their results as comparisons *between recipient groups*, not between donor and recipient groups (*1*, p. 400).
16. As Table 1 shows, the basic table in both experiments was a 3 by 2 contingency table. Although procedures for multiple 2 by 2 comparisons have been described, the authors apparently do not seem to be aware of the problems of partitioning contingency tables [T. A. Ryan, *Psychol. Bull.* **56**, 26 (1959); J. Bresnahan and M. M. Shapiro, *ibid.* **66**, 252 (1966)].

* Deceased 11 July 1971.

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Bryant *et al.* (*1*) have raised several criticisms of our own report (*2*). One of the major concerns seems to have been in regard to an erroneous chi-square analysis which appeared in one part of our report. Our error was acknowledged immediately after our report appeared, a correction statement was printed in *Science*, and a copy of the statement was sent to anyone requesting reprints (*3*).

The first issue discussed by Bryant *et al.* concerned the appropriateness of our passive avoidance paradigm in evaluating interanimal memory trans-

fer. It is well known that tasks which employ reaction time or latency data as dependent measures are subject to large intragroup variability. Traditionally, this problem has been dealt with by either refining the experimental situation, increasing the number of subjects, or employing a post hoc data transformation procedure. In our experiment, we considered the first alternative the most appropriate, and thus we find ourselves in complete agreement with Rosenblatt (*4*, p. 199) who suggested using negative reinforcement paradigms which have "the advantage that the rats need not be starved and consequently remain in better and more uniform condition throughout the experiment."

On their second point, Bryant *et al.* argue that a critical comparison in transfer studies should be based on the differences between untrained, or control recipients and recipients who received material from trained donors. The impression they attempt to create is that we failed to make the appropriate tests. In point of fact, however, almost all of our comparisons were among the different recipient groups. We therefore find this criticism puzzling.

Third, Bryant *et al.* claim that our choice of a 6-hour "recuperation" period after injections of homogenate was inadequate and also suggest we should have used more "objective" measures of recovery. We are somewhat surprised by their comment that 6 hours are inadequate since McConnell *et al.* have used this recovery period in several experiments (*5*, p. 132). We agree that objective measures of recovery from injections would have been more desirable, and we would have used them had they been available at the time our experiment was performed. Bryant *et al.* refer to two articles using objective criteria, but both have appeared since our experiments were published [references 4 and 9 in (*1*)]. It is important that even with the 6-hour recovery period, which may, or may not, have been adequate, we were still able to demonstrate significant differences among those animals receiving homogenates from trained or untrained donors. Bryant *et al.* suggest failure of recuperation, but do not give any reasons why subjects receiving homogenates from the trained donors should recuperate less rapidly than those receiving homogenates from the untrained. It is possible that Bryant *et al.* suspect the operation of either a two- or three-way interaction involving recovery period, tissue extract, or donor experience. While this remains an intriguing possibility, we are not aware of nor did Bryant *et al.* provide any evidence to substantiate this hypothesis. Bryant *et al.* next suggest that the amount of homogenate we injected may not have been adequate to produce a "true" transfer effect. This comment is paradoxical in light of Rosenblatt's (*4*, pp. 234 and 238) assertion that significant transfer effects have been obtained with as little as 0.0008 portion of brain. While convention might dictate the injection of extracts comprised of 1.5 to 2.0 brains as Bryant *et al.* claim, this would appear in view of Rosenblatt's data to amount to a case of overkill.

Bryant *et al.* also raise the possibility that the intense light we used in the white section of the alley may have served as an aversive stimulus in and of itself (and we agree with this observation). They suggest that this could account for the fact that nonshocked animals ran more rapidly into the dark portion of the apparatus. Unfortunately, their notion cannot account for why the subjects receiving homogenates from tumbled animals remained in the white compartment significantly longer than controls and could not explain our data on animals receiving liver homogenates. Thus, their attempt at a "reconceptualization" of the results from the nonshocked control group is not harmonious with all of our data.

The primary concern of Bryant *et al.* centers on our use of the Mann-Whitney test, the reliability of the liver recipient data, and our justification of the use of one-tailed significance levels.

1) As for one-tailed tests, we agree with Bryant *et al.* that our expressed rationale for using one-tailed criteria in the analysis of recipient latencies was misleading. A justification such as we employed, which was predicated on assumed donor-recipient differences, is untenable when testing for recipient-recipient differences. However, a donor-recipient comparison would not have been possible in our experiments since the "tumbled" donors were never placed in the shuttle runway and there were no latency scores obtained. Nonetheless, an inspection of our reported significance levels will indicate that the critical recipient-recipient differences persist under two-tailed criteria.

2) The comment by Bryant *et al.* of the use of the Mann-Whitney test is most puzzling. This test has been used in many transfer experiments (*6*) and has been specifically employed with one-tailed criteria by McConnell *et al.* (*5*, p. 250), Rosenblatt (*4*, p. 223), and Fjerdingstad *et al.* (*7*).

3) *Liver extract reliability*: Bryant *et al.* question the reliability of the effects which we obtained from recipient groups that were given liver homogenate injections from shocked or nonspecific stress donors. The basis of this concern is elusive since the outcomes were significant with either one- or two-tailed criteria. Furthermore, Essman and Lehrer (*8*) found similar results with liver extracts from donors trained to escape from a water maze.

Thus, there does seem to be some evidence that there are nonspecific transfer effects that may not be dependent upon RNA mechanisms. We stated this in our final paragraph as follows: "Since our studies were done with whole brain homogenates, no statement can be made regarding the effect of injections of pure RNA extract in interanimal transfer studies. We have shown, however, than an effect similar to that obtained with RNA can be demonstrated with stress-affected whole brain or liver substance" (*2*, p. 401).

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References and Notes

1. R. C. Bryant, A. M. Golub, J. V. McConnell, F. Rosenblatt, *Science* **178**, 521 (1972).
2. B. Frank, D. G. Stein, J. Rosen, *Science* **169**, 399 (1970).
3. Unfortunately, although our correction was submitted and accepted by *Science* in 1969, it did not appear in print until last August → *Science* **177**, 638 (1972)]. It should be noted that even with the corrected chi-square analysis, the pattern of results, and thus our conclusions, remained the same as in the original report. We again apologize for any inconveniences brought about by this error.
4. F. Rosenblatt, in *Molecular Approaches to Learning and Memory*, W. L. Byrne, Ed. (Academic Press, New York, 1970). Bryant *et al.* claim that the successful passive avoidance paradigm used by Ungar was substantially different from ours, yet in their reference 4 (*1*) his procedure appears very similar to ours and he reports a "high degree of reproducibility" with this method (p. 158).
5. J. V. McConnell, T. Shigehisa, H. Salive, in *Biology of Memory*, K. H. Pribram and D. E. Broadbent, Eds. (Academic Press, New York, 1970).
6. W. L. Byrne, in *Molecular Approaches to Learning and Memory*, W. L. Byrne, Ed. (Academic Press, New York, 1970), pp. xxiv–xxv.
7. E. J. Fjerdingstad, W. L. Byrne, T. Nissen, H. H. Røigaard-Petersen, in *Molecular Approaches to Learning and Memory*, W. L. Byrne, Ed. (Academic Press, New York, 1970), p. 161.
8. W. B. Essman and G. M. Lehrer, *Fed. Proc.* **25**, 208 (1966). We are surprised that Bryant *et al.* make no mention of their article in commenting on the reliability of liver "transfer" effect since Rosenblatt devotes a paragraph to a description and discussion of the Essman and Lehrer experiment in a recent review [F. Rosenblatt, in *Molecular Mechanisms in Memory and Learning*, G. Ungar, Ed. (Plenum Press, New York, 1970), p. 116].

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