Table 1. Comparison of performance in approach and avoidance situations for each cat following intramuscular injection of reserpine. All figures are percentages.

Responses	Cat							
	A	B	E	F	G	H	K	L
Avoidance								
Correct	29	47	67	71	29	82	53	31
Escape	10	25	4	18	10	8	8	21
No response	61	28	29	11	61	10	39	48
Pattern								
Correct	76	100	83	93	63	82	81	83
Error	9	0	0	7	12	0	4	3
Food only*	0	0	0	0	12	11	13	0
No response	15	0	17	0	13	7	2	14

* The cat approached and ate visible food but refused to approach patterns.

tioned stimulus was used as the index, it was found that reserpine affected the conditioned avoidance response to a visual cue much more severely than it affected the same response to an auditory cue. Friedman two-way analysis of variance (*3*) showed that this visual-auditory differential is significant at better than the 0.01 level. An extreme illustration of the differential is shown in Fig. 1. Since these responses were learned by two subgroups in counterbalanced order, it is possible to demonstrate that this differential susceptibility is not related to order of acquisition. It is related to the difficulty of learning the two responses, for avoidance to tone was acquired in fewer trials regardless of the order of learning. With sufficiently large doses of reserpine it was possible to block the avoidance responses to both stimuli, but examination of the time course of effects shows that the visually cued response is affected earlier and usually recovers later than the response to the auditory stimulus.

At times, when presentation of the conditioned stimulus did not elicit the conditioned avoidance response, a cat that had been injected with reserpine could be seen to cringe, growl, and sometimes attempt to escape from the box, on occasion even climbing to the ceiling of the compartment. These observations can be interpreted as evidence that the sensory mechanisms necessary for perception of the stimuli are functional and that the motor capacity of the animals to make the conditioned response is unimpaired. The adequate performance of a conditioned avoidance response to one stimulus but not to another, although the latter was followed by arousal and apparently affective reactions, suggests that the reserpine has not blocked what we might term the "anxiety-evoking potential" of the conditioned stimulus. The dosage levels used throughout these experiments were appreciably lower than those generally reported in current psychopharmacological investigations of reserpine, ranging from 7.5 to 65 μg/kg for intramuscular administration and from 25 to 300 μg total dose for central administration. Clear behavioral effects were demonstrated with doses as low as 7.5 μg/kg (intramuscular administration). The failure of Weiskrantz and Wilson (*4*) to find an analogous split between their approach and avoidance situations may be attributable to their excessively high doses (0.75 mg/kg) of reserpine, which resulted in a total behavioral depression.

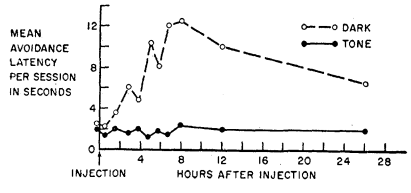

Fig. 1. Mean latency in seconds for avoidance responses to two different conditioned stimuli in one cat following central injection of 200 μg of reserpine (total dose).

Examination of the effects of reserpine on conditioned avoidance responses and on pattern discrimination for food shows a definite difference. Table 1 shows results based on 730 trials in shock avoidance and on 355 trials in pattern discrimination after intramuscular injection of reserpine (*5*). The two distributions of percentages of correct trials differ at slightly better than the 0.01 level, according to the sign test (*3*). This difference is not related to ease of acquisition in terms of trials to criterion. All cats showed such a split in behavior regardless of whether they learned pattern discrimination more or less quickly than they learned avoidance. These data suggest that reserpine affects behavior acquired under motivation from punishment more readily than it affects behavior reinforced by reward. It is true that the responses differ along other dimensions than that of approach versus avoidance but the data of Grastyán *et al.* (*6*) suggest that this may represent a basic physiological dichotomy (*7*).

The retention, under reserpine, of a visually mediated approach response simultaneously with the blocking of a visually cued avoidance response further demonstrates that the visual pathways remain functional during the period in which the animal is affected by reserpine.

It is felt that the data cannot be reconciled with the interpretation that reserpine at these doses blocks the conditioned avoidance response by interference with sensory perception, with motivation to perform, or with motor coordination. Therefore we propose that this selective action of reserpine may be attributed to an interference with the specific conditioned association between the stimulus and a directed evasion response—that is, interference with learned associations (*8*).

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References and Notes

1. Detailed reports on these studies are in preparation.
2. We wish to express our thanks to Dr. Jurg Schneider of Ciba Pharmaceutical Products Inc. for supplying us with reserpine (Serpasil).
3. S. Siegel, *Nonparametric Statistics for the Behavioral Sciences*, (McGraw-Hill, New York, 1956).
4. L. Weiskrantz and W. A. Wilson, Jr., *Ann. N.Y. Acad. Sci.* 61, 36 (1955).
5. Comparable data from central injections are not given here because of their sporadic nature, which is due to the fact that the placebo material in which reserpine is dissolved tends to destroy appetite when it is centrally injected. We were not aware of this phenomenon until we injected the placebo alone.
6. E. Grastyán *et al.*, *Kisérletes Orvostudomány* 9, 88 (1957).
7. Experiments are in progress to clarify whether or not it is the motivational dimension which is crucial to this distinction.
8. The research reported here was carried out with the support of the National Science Foundation (grant No. G-3354).

5 August 1957

Colorimetric Assay for Dipicolinic Acid in Bacterial Spores

Dipicolinic acid (pyridine 2,6-dicarboxylic acid) is a major component of bacterial spores (*1, 2*) and is unique in that it has been found only in such spores. It is synthesized during sporulation. The spores release dipicolinic acid during germination (*1*), or upon hydrolysis (*2*) or heating (*3*). Methods of analysis so far published (*2, 4*) are based upon ultraviolet absorption of the compound after acid digestion of the spores, isolation of the dipicolinic acid by ether extraction, paper chromatography of the extract, and elution of the acid-bearing spots. Though accurate, this method is extremely time-consuming and laborious. This report (*5*) describes a more convenient colorimetric method that

utilizes the color complex formed by interaction of ferrous iron with dipicolinic acid (*6*).

The complex of ferrous iron and dipicolinic acid which is prepared from the authentic dipicolinic acid described by Black, Depp, and Corson (*7*) is unstable, but the addition of a reducing agent such as ascorbic acid yields a complex that is stable for at least 2 hours. Maximum color develops in a *p*H range of from 4.0 to 6.0. In more acidic solutions, the color is diminished, and in slightly alkaline solution, precipitation occurs. Figure 1 shows the linear relationship between the optical density at 440 mμ and concentrations over a range from 30 to 160 μg/ml.

For the colorimetric assay of dipicolinic acid in spores, 5 ml of a spore suspension of *Bacillus cereus* (ATCC 10702) (in a 15- by 125-mm test tube) containing 4 to 20 mg of spores (dry weight) is autoclaved for 15 minutes at 15 lb/in^2. The suspension is cooled, acidified with 0.1 ml of 1.0*N* acetic acid, and left at room temperature for 1 hour, during which time aggregation of the insoluble material occurs. Upon centrifugation at 1500*g* for 10 minutes, a clear supernatant is obtained. If the initial suspension contains an appreciable number of vegetative cells, it may be necessary to increase the amount of acetic acid in order to obtain a clear solution. Four milliliters of supernatant are carefully pipetted into a clean 15- by 125-mm test tube. With a pipette controller, the extract can easily be removed without disturbing the sediment. One milliliter of freshly prepared reagent, consisting of 1 percent of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 1 percent of ascorbic acid in 0.5*M* acetate buffer at *p*H 5.5, is added to the 4 ml of supernatant. The color develops immediately, and the optical density should be measured within 2 hours. The blank is a similarly treated suspension in which 1 ml of water is substituted for the color reagent. The reagent blank is negligible. The standard is prepared by adding 1 ml of the color reagent to 4.0 ml of an aqueous solution containing 100 μg of dipicolinic acid per milliliter.

Determinations of dipicolinic acid by ultraviolet absorption and by colorimetric methods gave results which were in close agreement. Free dipicolinic acid was measured in an aqueous suspension of dried spores, as described by Krishna Murty and Halvorson (*4*). An ether extract of the suspension was chromatographed on paper, the spots containing dipicolinic acid were eluted, and the ultraviolet absorption of the eluate was measured at 270 mμ, with a Beckman DU spectrophotometer. This assay showed 41.6 μg of dipicolinic acid per milligram of spores. An aliquot of the same spore suspension assayed by the colorimetric method showed 42.3 μg of dipicolinic acid per milligram of dried spores.

Although divalent iron will react similarly with other α-carboxy pyridine compounds (*6*), the agreement in results between this method and the ultraviolet absorption method, which includes isolation of the dipicolinic acid, indicates that, for spore preparations, the assay is specific. Furthermore, no other α-carboxy pyridine compounds have been reported in spores. The addition of authentic dipicolinic acid to vegetative cell preparations yielded quantitative recovery of the dipicolinic acid upon assaying by the colorimetric method. This shows that vegetative cells contain neither components that interfere with the assay nor nonspecific compounds that assay as dipicolinic acid.

Other ions, including manganese and calcium, will complex with dipicolinic acid, but in the preparations studied no effective competition of these ions with the ferrous iron has been observed. In preparations in which the concentration of these ions is excessive, the interference can be overcome by increasing the concentration of the iron in the color reagent.

Autoclaving was found to release essentially all the dipicolinic acid of the spores. By colorimetric assay, we compared the dipicolinic acid released from a preparation of dried spores by autoclaving with that released by acid digestion in 3*N* HCl for 15 minutes. The amounts were 42.3 and 43.0 μg/mg, respectively. When the washed residue of the autoclaved suspension was digested in acid, an additional 0.45 μg of dipicolinic acid was recovered.

The data for the standard curve (Fig. 1) were obtained by means of a Beckman DU spectrophotometer, but all routine colorimetric assays were made with a line-operated spectrophotometer. The local line fluctuations were such that the probable error of replicate optical density readings at times exceeds 2 percent.

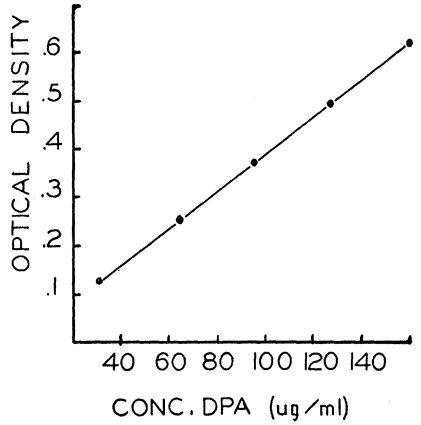

Fig. 1. The relation between optical density and dipicolinic acid concentration. Optical density was measured at 440 mμ with a Beckman DU spectrophotometer.

Under such conditions, no significance can be ascribed to the differences in results obtained with the two methods or to the trace of dipicolinic acid observed on the residues from the autoclaved preparation in the last experiment described.

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5. This report is Hormel Institute publication No. 161. This work was supported in part by research grant E-792 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, U.S. Public Health Service.
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23 September 1957

Evidence for a New Growth-Promoting Acid Produced by Lactobacillus casei

Lactobacillus casei 280-16 (or 280-16A) requires either D-lactic acid or any one of a number of other D-α-hydroxy fatty acids for optimal growth (*1*, *2*). Yeast extract serves as a source of D-α-hydroxy acid for this organism, and at least part of its nutritional activity is attributable to D-lactic acid (*1*). Culture filtrates of *L. arabinosus, L. casei* (hydroxy acid-independent strain), *Leuconostoc mesenteroides, Lactobacillus brevis,* and *L. fermenti* have also been shown to provide growth promoting material assumed to be D-lactic acid (*1*). The present investigation (*3*) reveals, however, that the growth stimulant to be found in cultures of *L. casei* is not D-lactic acid, but a more lipophilic acid, possibly a higher homolog of D-lactic acid.

Lactobacillus casei 280-16A and its hydroxy acid-independent parent (American Type Culture No. 7469) were incubated at 35°C for 72 hours in a medium free of hydroxy acid. The hydroxy acid deficiency of this medium was overcome, in the case of the hydroxy acid-dependent strain, by employing a very heavy inoculum, yielding an initial population of approximately 3× 10^8 cells per milliliter of medium. The nearly normal growth which resulted under these conditions was probably due to D-α-hydroxy acids carried either in or on the washed cells. An equally dense initial population was introduced in the case of the hydroxy acid-independent strain, al-