thus far, these antibodies disappeared as early as 6 to 10 weeks after inoculation.

(4) Rhesus monkeys inoculated intracerebrally with the homologous *mouse-adapted* Hawaii virus, developed C-F antibodies in titers as low as 1:16 which in some monkeys disappeared as early as 6 weeks after inoculation, although neutralizing antibodies in high titer persisted for many months.

(5) Human volunteers inoculated with dengue vaccine, consisting of the living mouse-adapted Hawaii virus, who developed neutralizing antibodies and resisted infection with the unmodified, human virus[1,7] *failed* to develop C-F antibodies.

(6) Sera obtained from individuals 2 years after a natural attack of dengue in Hawaii or Japan, which neutralized the Hawaii virus in high titer, also fixed complement in titers of 1:16 to 1:64. Positive results also have been obtained with sera of individuals who had dengue in Singapore, Java, and New Guinea.

(7) Sera collected from American marines, 11 months after a single, primary natural attack of dengue on Guam, gave positive C-F tests in titers of 1:2 to 1:8 with the Hawaii antigen, although they had no significant neutralizing antibodies[7] for this virus.‡

(8) Human volunteers infected with phlebotomus (pappataci, sandfly) fever virus developed no C-F antibodies for the dengue antigen.

(9) Rhesus monkeys which had no C-F antibodies for dengue 7 weeks after inoculation with the French neurotropic yellow fever virus, developed such antibodies in titers of 1:4 to 1:32, 8 to 10 days after a large booster dose of either the Asibi viscerotropic or French neurotropic strains of yellow fever virus.§ These results indicate an antigenic, if not an immunogenic,[1] relationship between the viruses of yellow fever and dengue.

[7] Sabin, A. B., and Schlesinger, R. W., unpublished studies.

‡ We are indebted to Drs. R. E. Shope and Horace Hodes, at the time serving with the U. S. Naval Medical Research Unit No. 2, for obtaining, lyophilizing and forwarding these sera in 1945.

§ We are indebted to Dr. Max Theiler, of the International Health Division of the Rockefeller Foundation, for the sera from these monkeys.

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Influence of Choline, Cystine and Methionine on Toxic Effects of Pyridine and Certain Related Compounds.

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In a study of the pharmacological effects produced by the ingestion of various pyridine derivatives by the rat[1,2] pyridine and coramine (nikethamide) were found to be the most active from the standpoint of the production of marked changes in liver size and composition. Coramine produced a great increase in liver weight without causing very much damage to the liver or modifying its composition. Pyridine has proved to be somewhat more toxic than many of its derivatives[1,3] and it must be included in the list of substances known to produce severe liver and kidney damage.[4,5] Methionine and cystine have been shown to have some liver protective

[1] Coulson, R. A., and Brazda, F. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 1.

[2] Brazda, F. G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 37.

[3] Brazda, F. G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 19.

[4] Baxter, J. H., *Proc. Am. Fed. Clin. Research*, 1945, **2**, 77.

[5] Baxter, J. H., *Am. J. Path.*, 1948, **24**, 503.

action when included in the diet of rats receiving pyridine.[4,6]

This report deals with the quantitative effects of the daily ingestion of pyridine or several heterocyclic compounds belonging to the quinoline series. The influence of various liver protective agents is also reported.

Experimental. The experimental animals consisted of equal numbers of male and female rats from our stock colony. The rats were placed on the test or control diets a few days after weaning. The basal diet had the same composition as that used for the coramine studies.[2] This diet consisted of 25% casein, 55% starch, 15% cottonseed oil and 5% salts. Five hundred ml of boiling water were added to each kg of dry diet. Each rat was given one ml of brewer's yeast extract a day and 3 drops of Percomorph oil a week.

The livers and kidneys were removed, weighed, and a small section was saved for histological examination. The livers were dried *in vacuo* and fat and water contents were determined as described by Brazda and Coulson.[2] Every effort was made to obtain the livers and kidneys for analysis from rats that had become moribund and had succumbed to the effect of the more toxic diets. Results obtained from these animals were so nearly alike regardless of the length of time the animals were on the diet that the data of all the moribund rats in a given experiment were gathered together into one group. In order to eliminate the effects of postmortem changes in the composition of the liver the tables presented do not include the results from any animal that had been dead for over 10 minutes. Records were kept of the growth rate, the food consumption, and length of life of the experimental animals.

In all, about 1200 rats were placed on diets containing pyridine or quinoline (in the form of acetates) to which various supplements suspected of being liver protective were added. Each rat received a fresh lump of the experimental diet each day and any diet left over from the previous day was discarded. The animals were weighed at the beginning of the experiment and at the time of death or at the time they were killed for autopsy. In all cases the experiments were concluded at 28 days. The experiments were conducted at an average temperature of about 75°F. As the season progressed the animals were removed to a room where the temperature was maintained at 76°F and the relative humidity at 50%.

Pyridine Series. Each group contained 70 rats, one female and one male from each of 35 litters. As far as was possible each litter contributed rats to 3 experimental groups. Group I received a diet which contained 0.6% pyridine; group II 0.6% pyridine plus 0.5% choline; group III 0.6% pyridine plus 1% cystine; group IV 0.6% pyridine plus 1.2% methionine; group V 0.6% pyridine plus 0.6% beta picoline; and group VI 0.6% pyridine plus 1.0% nicotinamide. In spite of the unpleasant odor of the diet the rats ate it willingly. There was no great difference in either food consumption or growth rate in the first 4 groups. The addition of beta-picoline or nicotinamide greatly reduced food intake which in turn reduced the growth rate. Two other pyridine groups were set up to determine the reproducibility of the survival curves. These groups are not included in Table I since there was no significant variation in the shape or the absolute area contained within the curve and hence it was concluded that 70 rats were sufficient for testing the effect of pyridine on survival.

The results are summarized in Fig. 1. Probably the best index of the relative survival time is obtained by comparing the respective areas under the curves. Considering a control curve to have an area of 1000, then the pyridine curve has an area of 348, the pyridine plus choline 465, the pyridine plus cystine 706, and pyridine plus methionine 773. It is apparent that methionine and cystine are far more effective in prolonging the life of the experimental animals than choline in spite of the fact that choline is known to be a lipotropic compound whereas cystine is ineffective in this respect. These results are in agreement with those reported by Baxter.[6] The survival curves of the groups receiving supplements of beta-picoline or nicotinamide in addition to the pyridine are not included in

[6] Baxter, J. H., *J. Clin. Invest.*, (*Proc.*), 1946, **25**, 908.

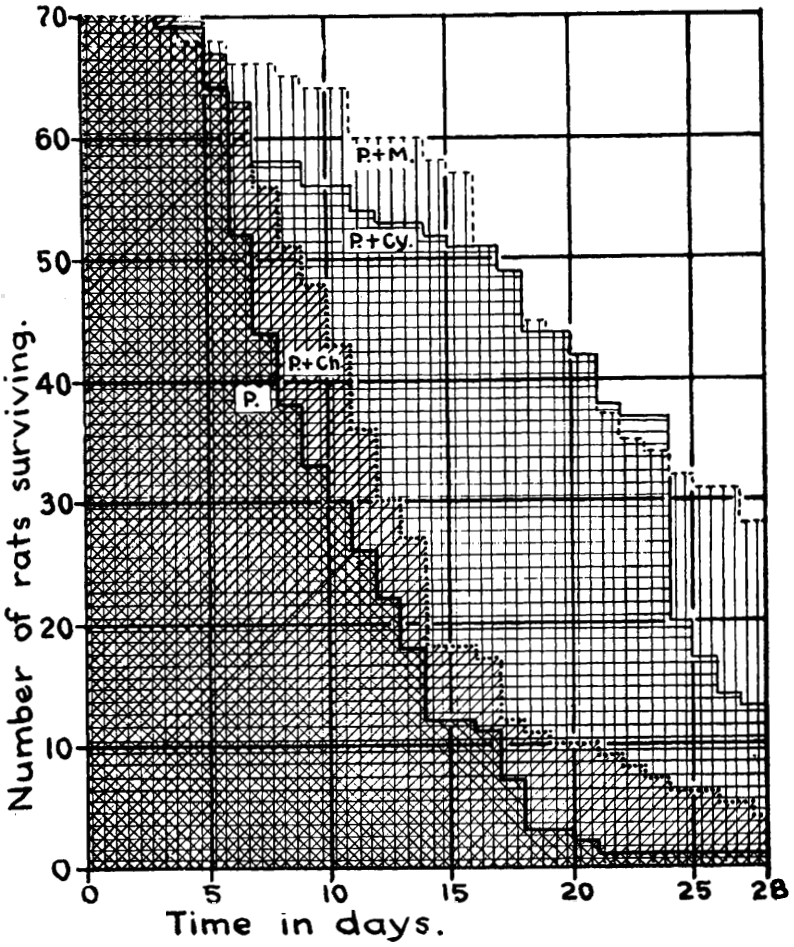

FIG. 1.

Survival of Rats on Pyridine Diets. P = 0.6% pyridine; P + Ch = 0.6% pyridine plus 0.5% choline; P + Cy = 0.6% pyridine plus 1.0% cystine; P + M = 0.6% pyridine plus 1.2% methionine.

Fig. 1. Both groups of animals ate much less and grew at a correspondingly lower rate which introduced the factor of food consumption. The area under the curves was greater than that found in the pyridine or pyridine and choline groups but much less than that found in the pyridine and cystine or pyridine and methionine groups. Paired feeding experiments are necessary to evaluate the influence of these two compounds since a decreased food intake means a correspondingly decreased intake of pyridine.

Table I presents the results of the liver analyses for the 6 groups of 70 rats each and for 10 rats which were fed piperidine, the reduced derivative of pyridine. The table may be summarized by stating that all of the rats in the pyridine experiments had marked liver enlargement. Without exception the moribund rats had livers which were larger than those of the sacrificed animals and which had a greatly decreased solid content. There are no significant differences in the growth rate of the animals receiving pyridine alone of those receiving additional supplements of choline or methionine. The cystine series grew at a rate somewhat below that of the others. Although the food intake and the growth rate paralleled each other rather closely both were subject to considerable individual variation.

The average fat contents of the pyridine

TABLE I.
Effect of Various Compounds on Size and Composition of Livers of Rats Receiving Pyridine.

Group	No. rats	No. days	Liver wt as % body wt		Liver fat, % dry wt	Liver % solids, avg	Gain in wt, g/day
			Avg	P.E.*			
0.6% pyridine—*Sacrificed*	11	5	6.21	±0.25	17.36	31.26	+1.42
	10	9	6.39	±0.12	21.33	33.05	+2.33
0.6% pyridine—*Moribund*	12	—	8.96	±0.24	24.14	25.08	—
0.6% pyridine + 0.5% choline—*Sacrificed*	10	5	5.84	±0.18	13.24	29.38	+1.81
	13	9	5.79	±0.12	11.49	28.23	+1.99
	2	28	5.57	—	11.76	31.03	+2.15
0.6% pyridine + 0.5% choline—*Moribund*	18	—	8.41	±0.17	12.60	22.91	—
0.6% pyridine + 1% cystine—*Sacrificed*	12	28	9.75	±0.33	16.23	26.52	+1.31
0.6% pyridine + 1% cystine—*Moribund*	5	—	10.33	±0.31	13.03	24.53	—
0.6% pyridine + 1.2% methionine—*Sacrificed*	11	5	5.89	±0.11	14.89	31.29	+1.13
	12	9	7.03	±0.16	16.17	28.75	+1.66
	28	28	6.50	±0.11	10.38	29.00	+1.97
0.6% pyridine + 1.2% methionine—*Moribund*	10	—	9.50	±0.13	12.14	22.91	—
0.6% pyridine + 0.6% beta picoline—*Sacrificed*	9	28	8.77	±0.25	17.19	31.11	+1.18
0.6% pyridine + 0.6% beta picoline—*Moribund*	7	—	9.44	±0.37	21.02	24.69	—
0.6% pyridine + 1.0% nicotinamide—*Sacrificed*	17	28	7.96	±0.31	14.78	28.70	+0.57
0.6% pyridine + 1.0% nicotinamide—*Moribund*	7	—	10.44	±0.24	16.47	25.38	—
0.6% piperidine	10	28	4.93	±0.14	7.59	28.46	+1.30
Control	10	5	4.98	±0.09	13.27	29.21	+3.21
	10	9	4.78	±0.09	13.38	29.15	+3.30
	10	28	5.18	±0.13	10.89	29.48	+3.79

* P.E. $= 0.6745 \sqrt{\frac{\Sigma(v)^2}{n(n-1)}}$

TABLE II.
Effect of Injection of a Lethal Dose of Pyridine on the Water Content of the Rat Liver.

Group	No. rats	Final wt avg	Liver wt as % body wt		Liver fat, % dry wt	Liver % solids, avg
			Avg	P.E.		
Experimental	18	49.5	4.90	±0.11	8.66	25.40
Controls	18	57.6	4.78	±0.17	14.62	32.60

and pyridine plus cystine groups are slightly higher than those of the groups receiving the lipotropic factors choline or methionine. The high protein diet is instrumental in preventing an excessive accumulation of fat in the livers of all groups. Piperidine had no noticeable effect on the liver although it did decrease food intake and growth rate.

Nine males and 9 female rats whose average age was 25 days were injected subcutaneously with pyridine in amounts that were calculated to be 3 times the LD_{50}.[3] These animals died in from 1 to 4 hours and the livers were removed, analyzed, and a piece was saved for histological study. The results appear in Table II. No significant liver enlargement was noted although the livers did show an increase in the average water content. The solid content was still considerably above the solid content found for the moribund group in Table I. In general the picture presented by the group injected with lethal doses of pyridine is quite different from that of the rats which were fed pyridine. It is probable these animals died from respiratory paralysis rather than from liver failure.

Quinoline Series. The survival curves found in Fig. 2 were obtained in the same manner as those of the pyridine experiments. Quinoline was used at a dietary level of 1% which is equivalent to 0.6% pyridine. If the control curve is given an arbitrary value of 1000 the relative area under the methionine curve is 812, the cystine is 745, the choline is 648, and the quinoline alone is 535. The fact that the relative order of effectiveness of the liver protective compounds is the same as in the pyridine groups suggests that the same mechanism is involved in both experiments. Methionine is again somewhat superior to cystine. Since the addition of choline does prolong the life of the quinoline rats it is probable that the slight difference between the pyridine and pyridine and choline groups in Fig. I is significant.

The results of the liver analyses are presented in Table III. The livers are very greatly enlarged in all 4 groups, approaching one-seventh of the total body weight. Although none of the rats presented in this table were moribund, the values for the liver solid content of the quinoline and the quinoline and choline groups closely resemble the figures obtained from the moribund rats of the pyridine groups. Without resort to histological examination it is apparent that cystine and methionine retard or prevent the hydration of the livers.

In the pyridine series it was observed that neither methionine nor cystine had any beneficial effect on the rate of growth of the animals on the diet reported in this paper. Quinoline alone retards the growth rate seriously and the addition of choline does not prevent this retardation. However the inclusion of either cystine or methionine increases the growth rate of the animals in a dramatic fashion. In the entire series of rats receiving quinoline with or without choline not one rat gained weight over the 28-day period. Of the 99 rats receiving quinoline plus cystine or methionine which survived long enough to be autopsied only 2 failed to gain weight. The growth rate was commensurate with the food intake which means that although those rats which received cystine or methionine ingested more quinoline, they remained in better health and lived longer than those of the other two groups.

A number of animals that had become moribund after eating diets which contained quinoline were examined immediately after death. It was not possible to obtain enough of these for the results to be of statistical significance. Those animals that died in the first few days had eaten so little of the diet that starvation

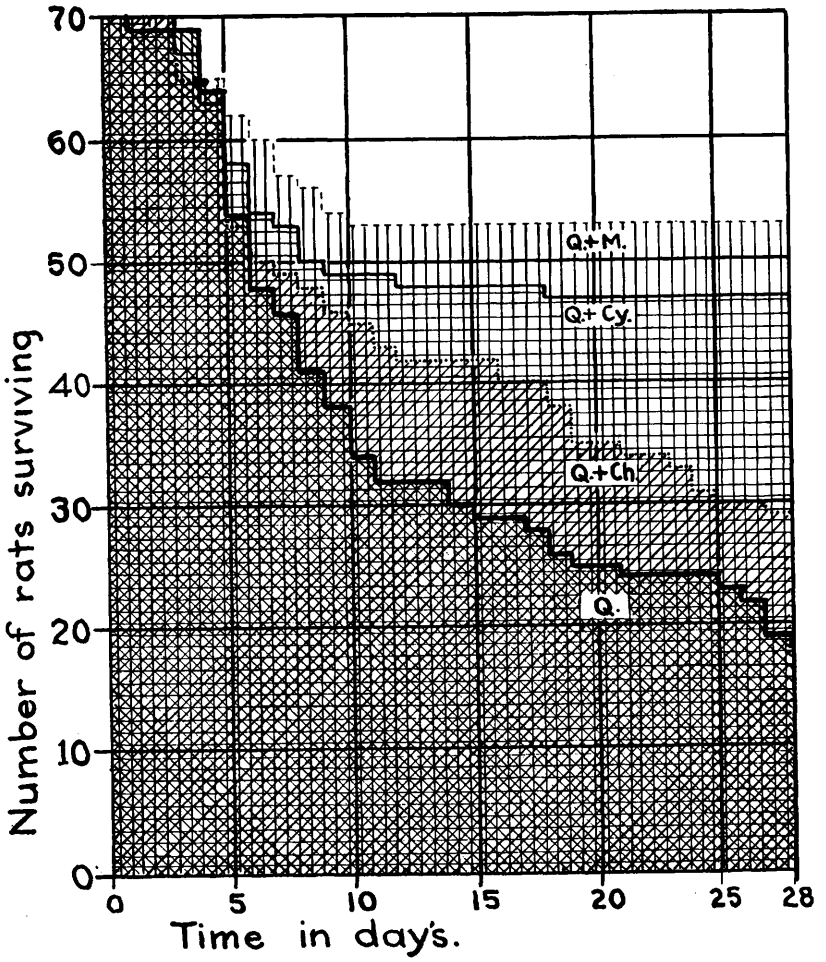

FIG. 2.
Survival of Rats on Quinoline Diets. Q = 1.0% quinoline; Q + Ch = 1.0% quinoline plus 0.5% choline; Q + Cy = 1.0% quinoline plus 1.0% cystine; Q + M = 1.0% quinoline plus 1.2% methionine.

probably contributed to a large extent to their death. In spite of this, gravimetric analyses of the livers of these few moribund rats gave results which were similar to those from the moribund animals of the pyridine groups. The very great increase in the liver weight of the sacrificed rats in the quinoline and quinoline and choline groups reported in Table III indicates that all of these experimental animals ate enough of the diet to produce liver damage.

On the basis of the theory that the tertiary nitrogen of quinoline is responsible for its action on the liver, quinoline was methylated to quinoline methochloride and this derivative was fed to 5 male and 5 female rats. The methochloride proved to be extremely toxic at a dietary level of 1.39% which is equivalent to a 1% quinoline level. Seven of the 10 rats died in less than a week; the other 3 were arbitrarily killed at 7 days and examined. The livers were normal in every respect and the experiments with this compound were not continued.

Isoquinoline at a level of 1% of the diet was also very toxic. Of the 20 rats that were fed this substance only 7 remained alive at 5 days. These 7 had livers which were hydrated to some extent although the degree of the liver enlargement was not marked. Isoquinoline methochloride was as toxic as isoquinoline but it produced no noticeable effect on liver size

TABLE III.
Effect of Choline, Cystine, or Methionine on Livers of Rats Receiving Quinoline.

Group	No. rats	No. days	Liver wt as % body wt		Liver fat, % dry wt	Liver % solids, avg	Gain in wt, g/day
			Avg	P.E.			
1.0% quinoline	18	28	11.27	±0.24	11.62	24.10	—0.40
1.0% quinoline + 0.5% choline	28	28	14.69	±0.10	18.21	25.92	—0.63
1.0% quinoline + 1.0% cystine	47	28	14.49	±0.13	14.49	29.69	+0.39
1.0% quinoline + 1.2% methionine	52	28	11.09	±0.16	11.15	28.55	+0.36

or composition.

Histological Appearance of the Livers. The ingestion of pyridine causes the production of cirrhosis or more commonly necrosis or both in the young rat. This is in agreement with results recently reported by Baxter.[5] On histological examination the livers of the moribund rats appeared to be hydrated and showed many small irregular vacuoles which were similar to those described by Trowell.[7] Gravimetric analyses of the whole livers, which usually reveal more information than does microscopic examination of small sections, clearly showed not only the presence of extra water but also the magnitude of its increase. In animals that died during the experiments with pyridine the histological picture of the livers was the same regardless of the presence or absence of a liver-protective supplement. The apparently healthy rats that were killed arbitrarily at different time intervals had livers which showed little damage. It would appear, therefore, that the development of severe necrosis following the ingestion of pyridine requires a relatively short period of time. Damage to the kidney is roughly but not absolutely proportional to the damage to the liver. Although many cases of kidney damage were seen few of these were severe enough to be considered as the primary cause of death and it was concluded that most of those animals that died from the ingestion of pyridine died from liver failure. Further evidence that liver failure was the cause of death may be obtained from consideration of the fact that all of the moribund rats had livers which were very severely damaged as shown by gravimetric analysis. The injection of lethal doses of pyridine into young rats had no effect on the histological appearance of the livers.

The animals of the quinoline series had livers which were more nearly normal than those in the pyridine groups as far as the microscopic picture was concerned. Although the cells were swollen and some small areas of necrosis were seen the overall appearance of the liver was not suggestive of marked damage. The percentage liver weights were from 2 to 2½ times the control weights, yet the extent and nature of the enlargement was not apparent from the slides. It is by no means certain that rats which died while receiving quinoline in the diet died of liver failure.

Discussion. The survival curves are useful in determining the toxic effects of compounds of the pyridine or quinoline nucleus and the influence of certain agents that reduce the toxicity of these substances. The sulfur containing aminoacids, methionine and cystine, are superior to choline in preventing liver damage. The fact that cystine is a good protective agent suggests that transmethylation is probably not involved in this protection since there is no evidence that cystine can contribute anything to methylation of the ring nitrogen as a means of detoxication. Although pyridine and quinoline may be methylated by the rat there is still no conclusive evidence that this is the case.[1] It is possible that the sulfur-containing compounds are involved in the detoxication of quinoline and pyridine in a manner as yet unknown.

Trowell[7] has reported that intact rats whose livers have been subjected to procedures designed to produce anoxia without reduction

[7] Trowell, O. A., *J. Physiol.*, 1946, **105**, 268.

of the intrasinusoidal pressure develop watery vacuoles in this organ. Inasmuch as the liver in a rat dying from effects of pyridine becomes hydrated, especially during the last hours of life, it seems logical to assume that this is due basically to a decrease in the rate of oxidation in the cells.

Summary. Pyridine alone or pyridine supplemented with either choline, cystine, methionine, nicotinamide or beta-picoline was fed to young rats on a high protein diet. Livers from rats dying from the effects of pyridine are enlarged and always exhibit a marked increase in water content. Livers from apparently healthy animals which have been on a pyridine diet show a nearly normal water content and are enlarged. Cystine and methionine afford protection against the effects of pyridine. The feeding of quinoline alone or quinoline supplemented with either choline, cystine, or methionine produced enlarged livers. Cystine and methionine increased survival time. Choline showed some protective effect. Cystine or methionine prevented the accumulation of water in the livers of rats fed quinoline. Choline had no effect.

16763

Quantitative Detection of Minute Concentrations of Digitoxin.*

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The detection of a digitalis glycoside, Lanatoside C, in various media by means of the embryonic duck heart preparation has been reported in previous studies.[1-3] It was found in these same studies that not only could extremely minute amounts of the glycoside (less than 0.1 μg per cc) be detected by this preparation, but also quantitative estimations of the actual amount of glycoside in Tyrode's solution or serum could be determined.

However, the sensitivity of the embryonic duck heart to the digitalis glucoside, digitoxin, had not been determined. Accordingly, studies were made of the effect of various concentrations of digitoxin upon the duck heart immersed in (1) Tyrode's solution, (2) rat serum, and (3) human serum. The method of quantitation employed was the same as that described previously.[1,3] It was found that the embryonic duck heart was not only extraordinarily sensitive to minute quantities of digitoxin but also offered a means whereby the concentration of digitoxin could be assessed in a quantitative fashion.

Results. As Table I demonstrates, the embryonic heart preparation was able to detect the presence of 0.005 μg of digitoxin in one cc of Tyrode's solution. Moreover, with increasing concentrations of the drug, there was a progressive reduction in the time taken for the occurrence of the digitalis effect.

The action of digitoxin in rat serum was found to be much less effective than in Tyrode's solution. Thus (Table I) only quantities of 0.2 μg or more of digitoxin per cc could be detected. Human serum was even more inhibitory (Table I) in that only quantities of 0.60 μg or more of digitoxin per cc could be detected. This action of serum upon the action of digitoxin also has been demonstrated by previous observers.[4,5] Lanatoside C, how-

* Aided by grants from the Life Insurance Medical Research Fund, The Public Health Service and The Sandoz Chemical Works.

1 Friedman, M., and Bine, R., Jr., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 162.

2 Friedman, M., and Bine, R., Jr., *Am. J. Med. Sci.*, 1947, **214**, 633.

3 Bine, R., Jr., and Friedman, M., *Am. J. Med. Sci.*, 1948, **216**, 534.

4 Fawaz, G., and Farah, A., *J. Pharm. and Exp. Therap.*, 1944, **80**, 193.

5 Suter, E., *Helvet. Physiol. et Pharmacol. Acta*, 1944, **2**, 2.