TABLE IV

CONSTANT SPECIFIC ACTIVITY OF URINE METABOLITES OF TRANYLCYPROMINE-C^{14} AND *dl*-AMPHETAMINE-C^{14} IN HIPPURIC ACID CARRIER

		c.p.m./μmole	
		Metabolite of tranylcypromine-C^{14}	Metabolite of *dl*-amphetamine-C^{14}
Consecutive Precipitations	(1)	2610	82.3
	(2)	2580	78.9
	(3)	2740	79.7
Suspension in petroleum ether		2670	76.8
Anilide derivative		2670	76.2

TABLE V

R_f OF URINE METABOLITES OF TRANYLCYPROMINE-C^{14} AND *dl*-AMPHETAMINE-C^{14} COMPARED WITH R_f OF CARRIER HIPPURIC ACID

Solvent[a]	Carrier hippuric acid	Metabolite of tranylcypromine-C^{14}	Carrier Hippuric acid	Metabolite of *dl*-amphetamine-C^{14}
I	0.82	0.85	0.84	0.85
II	0.50	0.51	0.50	0.52
III	0.65	0.65	0.62	0.64
IV	0.45	0.45	0.41	0.43

[a] See ref. 10.

Discussion

The identification of hippuric acid as a metabolite of tranylcypromine is the first demonstration that the cyclopropyl ring can be broken in the body. While the cleavage mechanism is unknown, it appears not to involve formation of amphetamine, since different patterns of peaks were observed in chromatograms of urine from rats given tranylcypromine-C^{14} and *dl*-amphetamine-C^{14} (Fig. 1 and 2). It is also unlikely that the first step is deamination by monoamine oxidase, since tranylcypromine has been recovered unchanged after incubation for 24 hr. in a rat-liver mitochondrial preparation containing the active enzyme.[11] This is in agreement with the view that monoamine oxidase is unable to oxidize α-alkylamines.[12] A liver microsomal enzyme system described by Axelrod[13] which deaminates such amines to ketones may be involved in the ring-splitting reaction. Another possibility is suggested from the propensity of tranylcypromine to decompose to ammonia and hydrocinnamaldehyde, upon standing in water solution for several weeks,[14] or upon distillation of the base.[15] In the body the aldehyde would be metabolized through hydrocinnamic, benzoic, and hippuric acids. Failure to identify hydrocinnamic acid in the present study would be explained by its rapid degradation to benzoic acid.[16] That an enzyme is involved in the cleavage reaction is suggested by the tested stability of tranylcypromine-C^{14} in water solutions buffered with phosphate at pH 7.8 and incubated at 37° for several days.

Identification of hippuric acid as a metabolite of amphetamine is evidence that the rat can degrade amphetamine, though this appears to be a minor pathway. The microsomal enzyme system described by Axelrod[13] deaminates amphetamine to phenylacetone, which is metabolized to hippuric acid in the body.[7] The small amount of hippuric acid-C^{14} recovered in the present study may be due to the low enzyme activities observed by Axelrod in rat liver as compared with rabbit liver. The present findings are in agreement with the view that the main metabolic pathway of amphetamine in the rat is not through deamination, as in the rabbit, but through ring hydroxylation with subsequent formation of the glucuronide.[13] The latter material probably accounts for the large peaks at the origins of the urine chromatograms shown in Fig. 1 and 2.

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Kinetics of the Formalin Inactivation of Poliovirus

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It is shown that the non-first-order course of the formalin inactivation of poliovirus is consistent with the kinetic requirements of a mechanism of the type, $N_n \rightleftharpoons nN \rightarrow nD$. In this formulation, N_n represents a non-infectious *n*-meric form of the virus, N is the infectious native form of the virus, and D is an antigenic but non-infectious species. The method used for testing the applicability of this mechanism is discussed in some detail. Several respects in which the postulated mechanism is related to previous proposals are also examined, especially the possible role of a superimposed thermal denaturation.

It is customary[1] to describe the time course of the denaturation of proteins, including viruses,[2,3] by a semi-logarithmic plot of protein concentration *vs.* time. Such denaturations are usually carried out in the presence of a large excess of denaturing agent, so the frequent linearity of plots of this type suggests a straightforward conversion of the protein to its denatured form with a pseudo-first-order mechanism for the rate-

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determining step. Treatment of the data in this manner has given linear plots for a variety of proteins.[1,4,5] In a number of cases, however, nonlinear plots result,[6–14] leaving in doubt even the grosser features of the mechanism by which the denaturation occurs. It is partly for this reason that the collective history of the experience of many laboratories with the formalin–poliovirus system has been a controversial one.[14–18]

A resolution of the kinetic features of the *in vitro* inactivation of poliovirus to some rational, and preferably linear, basis is of practical importance to vaccine production because of the need for destroying pathogenicity with minimum sacrifice of antigenicity.[18] The kinetic parameters of the formalin–poliovirus system have indeed been linearized, but only by ignoring the early part of the reaction[16] or by empirical techniques[14] that provide no mechanistic insight.

Woese[19] has shown that the kinetics of the formalin–poliovirus system are consistent with a thermal denaturation model, but alternative mechanistic tests have apparently not been applied with any reported success. It is the purpose of this report to show that other criteria are indeed applicable; the resulting interpretation, while technically at variance with that of Woese, at least agrees with his in suggesting that a one-step first-order mechanism for the reaction is unrealistic, both in principle and in fact. In this respect, both interpretations offer theoretical support for the intuitive conclusions already expressed by Timm, *et al.*,[15] by Stokes,[20] by Gard,[14] and by others.[18,21,22]

Wright and Schomaker[9] have studied the rate of inactivation of diphtheria antitoxin by urea in terms of a postulated mechanism of the type $P \rightleftharpoons N \rightarrow D$ and have found that the kinetic requirements of this mechanism are met to a precise degree by the experimental data. In this formulation, P is considered to be an inert, "protected" form of the active antitoxin N. An initially rapid drop in the measured titer of N accompanies the *de novo* establishment of reservoir P or is due to rapid inactivation of a pre-existing equilibrium concentration of free N; the remaining reservoir P is in either case then depleted *via* N at a slower rate, whose only measure is the variation of the concentration of N with time. The net result is a composite of three simultaneous reactions, similar in disappearance rate of the quantity measured to the over-all time course of the inactivation of poliovirus, depicted in Fig. 1 and reported *in extenso* by Timm, *et al.*[15] Chase[10] has deduced the same mechanism for the inactivation of *Cypridina luciferase*, but with more certainty that species P represents an inert form than expressed by Wright and Schomaker. Johnson, *et al.*,[23] have discussed this mechanism and have commented on the relative difficulty of establishing the inertness of P in the case studied by Wright and Schomaker.

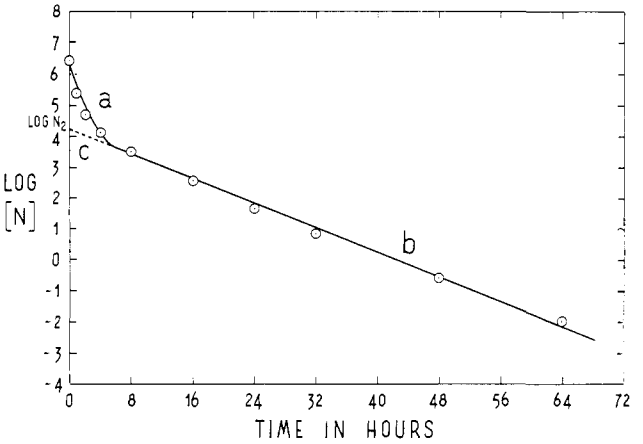

Fig. 1.—Inactivation of Type I strain pools of poliovirus by 1:4000 formalin at 37° and pH 7. Each experimental point is a statistically derived composite of the individual points from 6 replicate experiments, as described in the text. The theoretical curve was fitted to the experimental points by the method described in the text and shown graphically in Fig. 3.

A mechanism formally identical with that postulated by Wright and Schomaker but unrelated to protein denaturation has been offered by Sapirstein, *et al.*,[24] to account for the rate of plasma clearance of intravenously administered creatinine. Some of the kinetic requirements and mathematical features of this mechanism have been discussed in general terms by Defares and Sneddon,[25] by Evans,[26] and by Johnson, *et al.*[23] These treatments agree that the entire course of the postulated mechanism cannot in theory be accounted for by a linear relationship between time and the logarithm of the titer of N.

The integrated rate equation

$$[N] = N_1 \exp(-\lambda_1 t) + N_2 \exp(-\lambda_2 t) \qquad (1)$$

which describes the concentration of N as a function of time for the process, has been derived from first principles.[9,23,25,26] The theoretical relationships between the concentration variables, [N], [P], and [D], that this mechanism predicts for the early part of the reaction are shown in Fig. 2, adapted from Evans.[26]

Experimental

Representative data for the formalin inactivation of poliovirus (*P. hominis*, Type I strain pools), obtained by previously published methods,[15] are summarized in Fig. 1; the experimental points shown were established by plotting the logarithm of the number of tissue culture infectious doses of virus per unit volume remaining after various times of exposure of the virus culture to the indicated concentration of formalin. The point at $t = 0$

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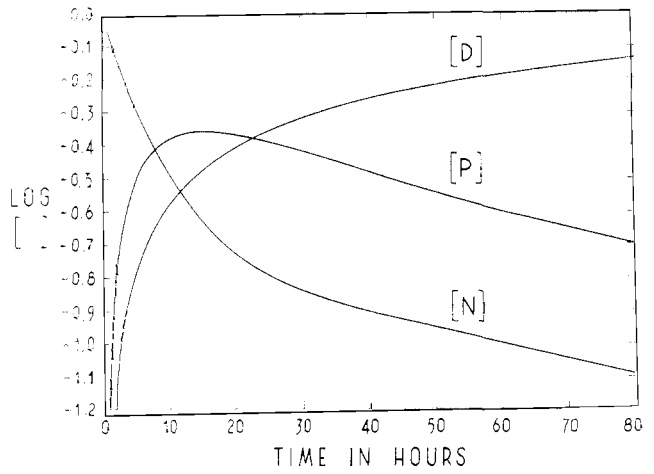

Fig. 2.—Theoretical relationships among concentration variables for the mechanism, $P \overset{k_2}{\underset{k}{\rightleftharpoons}} N \xrightarrow{k_3} D$, with $k_1 = 0.1t^{-1}$, $k_2 = k = 0.05t^{-1}$, $[P]_0 = 0$, $[D]_0 = 0$, and $[N]_0 = 1$. Adapted from Evans.[26]

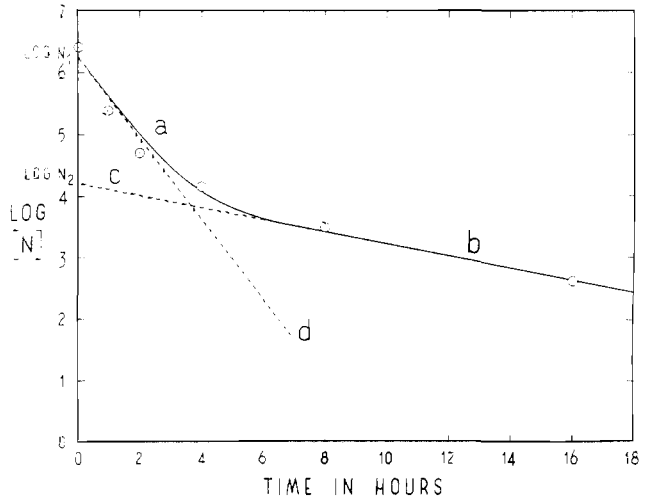

Fig. 3.—Larger scale version of the early part of Fig. 1 and the geometric construction upon which the fit shown there depends. In theory, segments a and d cannot meet, as suggested here, at the $t = 0$ axis; their *apparent* meeting is due to the difficulty of depicting the small difference between log [N] and log ([N] − N_2) at $t = 0$ on the scale chosen.

represents virus titer *before* addition of the formalin. The superimposed curve in Fig. 1 was fitted independently by a method to be described later. Each experimental point in Fig. 1 represents the geometric mean of the values of virus titer calculated after rejecting the upper and lower quartiles from 6 replicate experiments. Extensive experience in these Laboratories with many production lots of poliovaccine[15,18] has shown that the extreme deviation of a single experimental point from the mean rarely exceeds ±0.5 log unit.

A fit of eq. 1 to the data of Fig. 1, as a test of the proposed mechanism $P \rightleftharpoons N \rightarrow D$ can be achieved by a method[25] that avoids the more laborious least-squares technique used by Wright and Schoenaker[8]; the result is the curve connecting the experimental points in Fig. 1. This valuable test, which depends upon the geometric construction shown in Fig. 3, is little enough known to require some detailed comment, as follows.

Provided $\lambda_1 \neq \lambda_2$, no part of the locus of eq. 1 can, in theory, be converted to a linear form on semilogarithmic axes, since neither term can ever be zero; however, to the extent that the proposed mechanism applies, segment b in Fig. 1 approaches linearity as the term, $N_1 \exp(-\lambda_1 t)$, *approaches* zero and, in practice, a straight line can be visually fitted to segment b and extrapolated to the $t = 0$ axis, giving the line bc and intercept, log N_2. Segment a and the dotted extrapolation of segment b are shown to larger scale in Fig. 3. The resulting straight line, bc, in Fig. 3 has the form

$$\log z = -\lambda_2 t + \log N_2 \qquad (2)$$

or, converting to exponential form

$$z = N_2 \exp(-\lambda_2 t) \qquad (3)$$

In order to define the new variable z, a new line a, with intercept log N_1, is constructed by converting a series of synchronous points on a and segment a to a plot of log ([N] − z) vs. t on the same scale; the line resulting, *if straight*, has the form

$$\log([N] - z) = -\lambda_1 t + \log N_1 \qquad (4)$$

or, converting to exponential form

$$[N] - z = N_1 \exp(-\lambda_1 t) \qquad (5)$$

Substituting eq. 3 into eq. 5 gives the desired result, eq. 1

$$[N] = N_1 \exp(-\lambda_1 t) + N_2 \exp(-\lambda_2 t)$$

Thus, the straightness of d is a test of the applicability of eq. 1 to the entire curve ab in Fig. 1; this, in turn, is a test of consistency between the experimental data of Fig. 1 and the mechanism here proposed. Transference of the confidence limits of segment a to line d gives the desired measure of confidence in the straightness of the latter as a test of the mechanism in question.

Results

The range of biological variation of each experimental point in Fig. 1 is admittedly greater than ideal, but at face value the fit of theory to experiment shown there is excellent; well within the statistical limits imposed, as good as the fit relied upon by Woese,[19] and certainly better than is obtained by insistence upon a one-step first-order mechanism.

Discussion

Curves of the same general shape as that shown in Fig. 1 have been reported[15] for other types and strains of poliovirus examined under other conditions, so the postulated mechanism would appear to be more broadly applicable to this important area of virology than specifically indicated here. Weidenkopf's remarkably similar data[27] for the inactivation of poliovirus by chlorine suggest that the phenomenon is an inherent property of the virus and not an isolated peculiarity of formalin.

It should be emphasized that the above curve-fitting method serves simply to test the plausibility of the postulated mechanism and is *not* an indirect means for linearizing curve ab throughout its entire length. It is clear from Fig. 3, however, that segment a and straight line d are nearly colinear for the early part of the reaction; segment b is also approximately linear, so the entire curve ab can in practice be resolved into two approximately linear components with slopes λ_1 and λ_2. This indicates that, very early and very late in the course of the reaction, one or the other of the first-order exponential terms in eq. 1 is in effect rate-defining. Applicability of the proposed mechanism thus offers theoretical support for the contention of Charney, *et al.*,[28] that the safety end point in vaccine production can be approximated by extrapolating segment b across the minimal infectious dose baseline. This could be done with more confidence, of course, in the limiting case $\lambda_1 = \lambda_2$, for then eq. 1 collapses, as it should, to the

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integrated form of the simple first-order rate equation, $\log [N] = -\lambda t + \log [N]_0$ in which $[N]_0 = (N_1 + N_2)$, the $t = 0$ intercept. This behavior, though never seen in practice in these Laboratories, is more closely approached by some of the inactivation curves reported by Timm[15] for other conditions or other strains of virus than by the data of Fig. 1.

As a curve-fitting technique, the procedure described here is more convenient and no less valid when applied in reverse; thus line *c* is first found, line *d* then drawn, and finally segment *a* constructed. In either case, however, the result is a curve of visual best fit whose geometry expresses one of the requirements of the mechanism proposed here.

Protein denaturation of course cannot be as simple in reality as depicted here, and the present results certainly do not in themselves establish the physical existence of species P; they do show, however, that the system behaves "as though" P were real and not *per se* pathogenic. Charney's account[29] of the kinetics of inactivation of highly purified poliovirus is difficult to interpret in the light of later reports by Schaffer[22] and by Charney, *et al.*,[28] but the latter, at least, suggest also that the reaction proceeds as though P were real; the purity claimed by these authors could be only relative, however, and the relationship between protection of the type envisaged here and the presence of nonviral material in the cultures cannot be finally established without further, more decisive tests. It is, nevertheless, reasonable to suggest that the relationship, $N \rightleftharpoons P$, may be an inherent property of the virus or its response[18] to the presence of formalin, and independent of the presence of nonviral material; if so, then P may actually represent an *n*-meric micellar form of the virus, allowing a depiction of the proposed mechanism in the following more committal terms

$$N_n \rightleftharpoons nN \rightarrow nD$$

By either formulation, it is tempting to equate the protected form of the virus visualized here with the "clustered" or "masked" form suggested by other authors[17,18,30–32] as a possible explanation of the non-first-order course of the reaction. The reversible nature of the association between N and N_n could then account for Timm's observation,[15] designed as a test of the clustering premise, that inactivation curves are not linearized as expected by ultrafiltration; *i.e.*, by assuming that the virus passes through the filter as monomer, followed by re-establishment of the equilibrium in the filtrate. This equilibrium property could also explain the failure[15] of ultrasonic irradiation to rectify the subsequent response of virus cultures to formalin treatment.

Woese has shown[19] that inactivation data of the type presented here can also be accounted for by a thermal denaturation mechanism in which the presence of formalin is assumed to be noncontributory. The Woese mechanism is formally indistinguishable from the present one without further data, for the two mechanisms are not only remarkably similar as written, but their rate equations differ only in the significance of the four constant terms present in each.[9] It is hard to conceive of experimental circumstances under which a virus that is denaturable by heat alone could also be denaturable at the same temperature and approximately the same rate by formalin without a measurable thermal contribution: these ostensibly different mechanisms can be equated, however, by broadening the notion of "protection" to include thermal "insulation" or resistance[33] as well as protection of the infectious entity from formalin attack. Woese has correctly observed, however, that, while the two types of inactivation may not be mutually exclusive, a distinction between them or an assessment of their relative importance must await the availability of relevant thermodynamic data.

If denaturation by formalin and by heat do in fact occur concurrently and by the mechanisms depicted here and by Woese, then a full description of the reaction would require, in the most general case, a rate equation of the type $[N] = N_1 \exp(-\lambda_1 t) + N_2 \exp(-\lambda_2 t) + N_3 \exp(-\lambda_3 t) + N_4 \exp(-\lambda_4 t)$, in which two of the terms refer to thermal effects in the sense intended by Woese. The associated 4-phase curve could be fitted to a given set of data by repeating the procedure described earlier, with perhaps[25] even more accuracy than is apparent in Fig. 1; unfortunately, however, the possible role of coincidence would also become correspondingly greater with this increased multiplicity of terms.[25] The possibility of resolving inactivation curves into more than two discernible phases has been discussed by McLean and Taylor[18] with special reference to the possible role of thermal effects. A slight but systematic discrepancy between theory and experiment is noticeable in Fig. 1; this discrepancy, if real, could be taken as evidence that Fig. 1 is actually an oversimplification of a slightly more complex curve and that both types of inactivation do in fact occur simultaneously, but at slightly different rates.

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