[7] Gros, F., H. Hiatt, W. Gilbert, C. G. Kurland, R. W. Risebrough, and J. D. Watson, *Nature*, **190**, 581 (1961).
[8] Reddi, K. K., *Science*, **144**, 566 (1964).
[9] Kirk, J. T. O., *Biochim. Biophys. Acta*, **76**, 417 (1963b).
[10] Zaitlin, M., and N. K. Boardman, *Virology*, **6**, 743 (1958).
[11] Zech, H., and L. Vogt-Kohne, *Naturwissenschaften*, **42**, 327 (1955).
[12] Bald, J. G., and R. A. Solberg, *Nature*, **190**, 651 (1961).
[13] Hirai, T., and S. G. Wildman, *Plant Cell Physiol.*, **4**, 265 (1963).
[14] Watson, B. K., and A. H. Coons, *J. Exptl. Med.*, **99**, 419 (1954).
[15] Liu, C., *J. Exptl. Med.*, **101**, 677 (1955).
[16] Breitenfeld, P. M., and W. Shäfer, *Virology*, **4**, 328 (1957).
[17] Traver, M. I., R. L. Northrop, and D. L. Walker, *Proc. Soc. Exptl. Biol. Med.*, **104**, 268 (1960).

*DOUBLE-STRANDED RNA FROM TOBACCO LEAVES INFECTED WITH TMV**

BY W. SHIPP† AND R. HASELKORN‡

DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF CHICAGO

Communicated by William Bloom, June 24, 1964

Following the discovery by Sinsheimer and co-workers of a double-stranded, replicative form of bacteriophage ϕX174 DNA,[1] a number of investigators have endeavored to find analogous intermediates in the replication of viruses containing single-stranded RNA. An important tool for the isolation and identification of such RNA species, namely, their resistance to degradation by pancreatic RNase, was provided by Geiduschek *et al.*[2] Double-stranded RNA was also expected to have a lower buoyant density in Cs_2SO_4 than its single-stranded counterpart. With these properties in mind, we sought a virus-specific, double-stranded RNA in tobacco leaves infected with TMV.[3]

Similar investigations in animal and bacterial systems have been the subject of several recent reports. Montagnier and Sanders showed that a double-stranded RNA could be obtained from cells infected with EMC virus.[4] Their observations have been extended by others to poliovirus-infected HeLa cells,[5] and to *E. coli* infected with bacterial viruses MS2,[6, 7] fr,[8] and R17.[9] Tobey has reported on the absence of double-stranded RNA in Mengovirus-infected L cells treated with actinomycin D.[10] We present herewith evidence for the existence of double-stranded RNA in tobacco leaves infected with TMV.

Materials and Methods.—TMV-infected leaves: The growing tip and all but one of the lower leaves were cut from tobacco plants (*Nicotiana tabacum* var. Turkish Samsun) in the 10- to 15-leaf stage. The remaining leaf on each plant was infected with TMV, strain U-1. After 5–7 days, axillary buds have developed into small clusters of leaves. These leaves were harvested 10–20 days after infection, weighed, and frozen until used. In this way 10,000 systemically infected leaves are handled with only minor difficulty.

Double-stranded RNA: Crushed, frozen leaves (usually 2,000–5,000) were homogenized at room temperature in a Waring Blendor containing 25 ml of water-saturated, distilled phenol and 25 ml of an extraction suspension (2% SDS, 0.05 *M* EDTA, pH 8, and 0.5% bentonite) per 100 gm of leaf tissue. The homogenate was filtered through cheesecloth to remove debris. The debris was re-extracted with a small volume of phenol and extraction suspension. The filtered slurry was centrifuged, and the aqueous phase was removed and deproteinized further by three or more

phenol extractions. Phenol, some residual protein, and SDS were removed by extracting the material with chloroform and 1-octanol (24:1, v/v). After several such extractions, the nucleic acid was precipitated by the addition of two volumes of cold ethanol, and collected by centrifugation. The pellet was resuspended in 0.01 *M* Tris, 10^{-3} *M* Mg^{++}, 0.1 *M* NaCl, pH 7.5, and insoluble material removed by centrifugation (10,000 rpm for 10 min in a Servall centrifuge, model RC2). The supernatant was then digested with DNase (20 μg/ml, Worthington Biochemical Corp., crystallized pancreatic DNase, 1 hr, 27°C). The remaining material was deproteinized with phenol, extracted with chloroform-octanol, precipitated with ethanol, and resuspended in a small volume of 0.01 SSC and then adjusted to SSC. After digestion with RNase (10–100 μg/ml, Worthington, crystallized pancreatic RNase, lot no. R617, 1 hr, 27°C), 500 μg/ml of bentonite was added, and the material was deproteinized with phenol. Phenol was removed with chloroform-octanol. The aqueous phase was centrifuged (12,000 rpm, 10 min in Servall centrifuge) and then filtered through a Type HA Millipore filter (0.45-μ pore size) to remove any insoluble material. The filtrate (10–20 ml) was dialyzed against several changes of 2 SSC for 12–24 hr to remove RNase digestion products, and then lowered to 0.01 SSC by dialysis.

P^{32}-labeled viral RNA: Half-expanded leaves of tobacco plants were infected with 500 μg/ml of TMV. Twelve hours after infection, the leaves were cut from the plant and their petioles immersed in 1/10 strength Hoagland's solution no. 1[11] with carrier-free P^{32}-orthophosphate as the only source of phosphorus. After 10–14 days, virus was extracted from the plants and purified by differential centrifugation.[12] A similar procedure was used to label leaves when attempts were made to extract P^{32}-labeled double-stranded RNA from infected leaves. Viral RNA was prepared with phenol and bentonite according to Fraenkel-Conrat *et al.*[13] Labeled TYMV-RNA was prepared as described previously.[14]

Preparation of P^{32}-TYMV C-RNA:[15] The following were incubated at 30°C in a final volume of 5.0 ml: 600 μmoles Tris pH 7.5; 5 μmoles each of ATP, GTP, CTP; 0.5 μmoles UTP^{32}; 15 μmoles $MnCl_2$; 350 μg TYMV-RNA; and 2 mg of *M. lysodeikticus* RNA polymerase purified through step VI.[16] Although 1 hr is sufficient for maximum UTP incorporation, the particular preparation of TYMV-C-RNA used in this work was incubated for 2 days. The reaction mixture was incubated for 15 min at 36°C after the addition of 50 μmoles EDTA and 100 μg RNase. Deproteinization with phenol followed. The aqueous phase was then dialyzed against several changes of 0.5 *M* NaCl, and finally against 0.01 *M* Tris pH 7.5.

Annealing of double-stranded RNA with viral RNA: Samples of double-stranded RNA were heated for 10 min at 100°C in 0.01 SSC. P^{32}-labeled viral RNA in 2 SSC was added to the sample at 100°C. Sufficient 10 SSC was added to make the sample 2× with respect to SSC. The sample was then transferred to an 80°C bath and slowly cooled to 35°C over a 12-hr period. After annealing, the sample was cooled to 0°C and kept at this temperature until used.

Assay for RNase resistance of P^{32}-labeled RNA: Samples of less than 0.25 mg/ml of RNA in 2 SSC were incubated with 100 μg/ml RNase for 30 min at 27°C. The material was then cooled to 0°C, and 200 μg of yeast RNA was added as a carrier. After the addition of 1/9 volume of 50% TCA, the precipitate was collected on a Millipore filter (Type HA, 0.45-μ pore size), washed with a minimum of 30 ml of cold 5% TCA, and counted in a thin end-window Geiger counter.

Results.—In early experiments, using an RNase- and DNase-resistant (nuclease-resistant) nucleic acid fraction from infected leaves labeled with P^{32}, we sought to detect any double-stranded RNA by virtue of a possible difference in banding density in Cs_2SO_4. The density at which a species of RNA banded was found to be very difficult to reproduce if two or more RNA's were banded together. In some experiments, RNA was found which banded at a lighter density than TMV-RNA and leaf ribosomal RNA, which would be expected of a double-stranded molecule. However, when duplicate gradients of the same material were run, light material present in one tube was often missing in the other. The results of three replicate gradients are shown in Figure 1. Material lighter than TMV-RNA (1.63 gm/ml) and leaf ribosomal RNA (1.68 gm/ml and 1.63 gm/ml) does appear in the gradients. However, with such high backgrounds of host RNA, no conclusion can be drawn concerning the existence of a double-stranded species by virtue of its banding

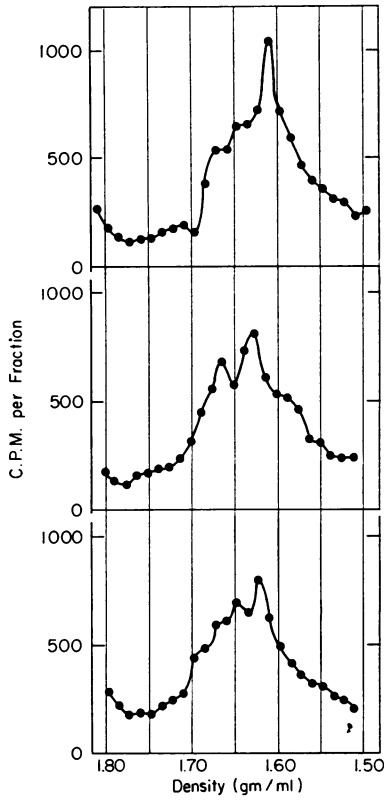

FIG. 1.—Cs_2SO_4 density gradient centrifugation of P^{32}-labeled, nuclease-resistant extract from systemically infected leaves. Each tube contained 5 ml with average density 1.6 gm/ml. Centrifugation was for 70 hr at 35,000 rpm at 25°C in SW 39 rotor of model L Spinco. The extract was prepared (see *Methods*) from leaves labeled for 76 hr starting 7 days after inoculation of the plant with TMV. RNA specific activity 5×10^6 cpm/mg. Each tube contained extract equivalent to 125 leaves.

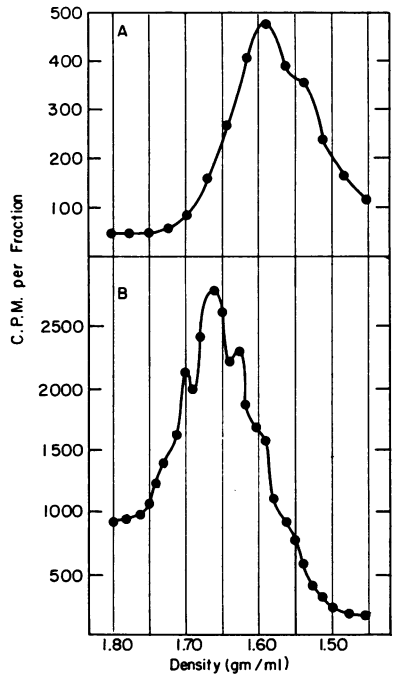

FIG. 2.—Cs_2SO_4 density gradient centrifugation of TYMV-C-RNA alone (*A*) and together with nuclease-resistant extract from TMV-infected tobacco leaves (*B*). Specific activity of the C-RNA was 47×10^6 cpm/mg. Centrifugation was for 90 hr at 35,000 rpm at 25°C in SW 39 rotor of model L Spinco. The extract in (*B*) contained 30 leaf equivalents, in this case less than 100 μg of RNA.

density. The base compositions of light RNA regions ($1.55 < \rho < 1.61$) from two different experiments were determined by alkaline hydrolysis. These gave A = 24, G = 40, U = 17, C = 18. Comparison with the base compositions of leaf ribosomal RNA, TMV-RNA, and a hypothetical double-stranded form containing one strand of TMV-RNA and one strand of its complement shows that the observed light bands are not identical in composition with any of these species. The high purine content of the light bands suggests that they consist predominantly of ribonuclease digestion products.

That such banding patterns are probably artifacts is shown in a reconstruction experiment using P^{32}-labeled TYMV C-RNA and unlabeled, nuclease-resistant leaf extract. Figure 2*A* shows the banding pattern of C-RNA alone. In Figure 2*B*, the C-RNA is seen to be aggregated with partially digested material and has banded at heavier densities.

In view of these difficulties, an alternative approach that offered a lower back-

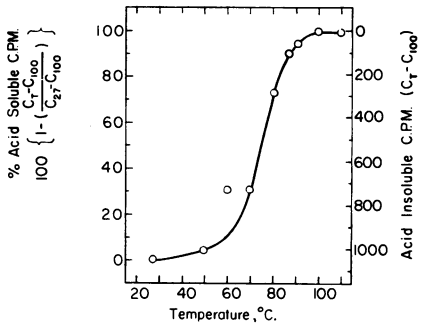

FIG. 3.—Thermal dissociation in 0.01 SSC of RNase resistance of P^{32}-TMV-RNA annealed with infected leaf extract. Aliquots of the annealed mixture were heated at the temperature indicated for 10 min and then cooled rapidly. Unlabeled TMV-RNA (200 μg) was added, the sample adjusted to 2 SSC, and digested with RNase (see *Methods*). Acid-precipitable cpm remaining after heating to 100°C and RNase digestion, designated C_{100}, were 650 cpm.

ground and greater sensitivity was tested. If double-stranded RNA, consisting of a viral RNA strand and its complement, is present in unlabeled, nuclease-resistant extracts, it should be possible to replace some of the unlabeled viral RNA in the duplex with labeled viral RNA *in vitro*. Such specific hybridization could be detected by measuring an increase in the RNase resistance of radioactivity after heating the extract and annealing with P^{32}-labeled viral RNA. This method has recently been successfully used to assay for the "replicative form" of MS-2 virus.[7] The results of one series of experiments are shown in Table 1. When the extract was heated to 100°C in 2 SSC and annealed with labeled RNA, very little increase in RNase-resistant radioactivity was observed. However, when the extract was heated in 0.01 SSC and annealed, 10.7 μg of the labeled viral RNA became RNase-resistant. To obtain maximum annealing, the labeled RNA had to be introduced

TABLE 1

CONFERRAL OF RNASE RESISTANCE UPON P^{32}-LABELED TMV-RNA BY A NUCLEIC ACID FRACTION FROM TMV-INFECTED LEAVES

Number	Treatment	Cpm above control	μg RNAse-resistant RNA
1	Heat extract in 2 SSC. Quench. Add TMV-RNA. Anneal in 2 SSC.	10	—
2	Heat extract in 0.01 SSC. Quench. Add TMV-RNA. Anneal in 2 SSC.	2600	5.6
3	Heat extract in 0.01 SSC. Add TMV-RNA at 100°C. Anneal in 2 SSC.	4990	10.7
4	As in 3. Dialyze out salt vs 0.01 SSC after annealing. Add cold TMV-RNA. Heat. Quench. Make 2 SSC.	90	<0.2
5	Hydrolyze extract. (0.1 *N* NaOH, 12 hr, 27°C). Neutralize. Dialyze vs 0.01 SSC. Treat as in 3.	0	—
6	Heat extract. Make 2 SSC at 100°C. Quench. Add TMV-RNA at 0°C. Allow to come to room temperature. (15 min)	905	1.9
7	Heat extract. Add TMV-RNA at 100°C. Make 2 SSC. Allow to come to room temperature. (10–15 min)	1550	3.2
8	Heat extract. Add TYMV-RNA at 100°C. Make 2 SSC. Anneal.	40†	<0.1
9	As in 8. Heat to 50°C in 0.01 SSC. Quench. Make 2 SSC.	21†	<0.1
10	Heat RNA from healthy leaves.* Add TMV-RNA. Make 2 SSC. Anneal.	0	—

* 3.65 mg of total leaf RNA prepared as extract, except no RNase treatment.

† Normalized to same specific activity as TMV-RNA.

0.9 ml of nuclease-resistant extract (51 leaf equivalents/ml; absorbancy = 10.5 at 260 mμ) was heated at 100°C for 10 min and quenched by rapidly cooling the solution to 0°C. 321 μg of TMV-RNA with a specific activity of 467 cpm/μg was added as described, making a final volume of 1.35 ml. See *Methods* for details of assay of RNAse-resistant radioactivity. Controls, consisting of viral RNA carried through the same heating and annealing procedures without extract, gave 1420 cpm for Expt. 4, 2190 cpm for Expts. 6 and 7, and 1820 cpm for Expts. 1, 2, 3, 5, 8, 9, and 10. Cpm are averages of two determinations, each counted to 10^4 cpm.

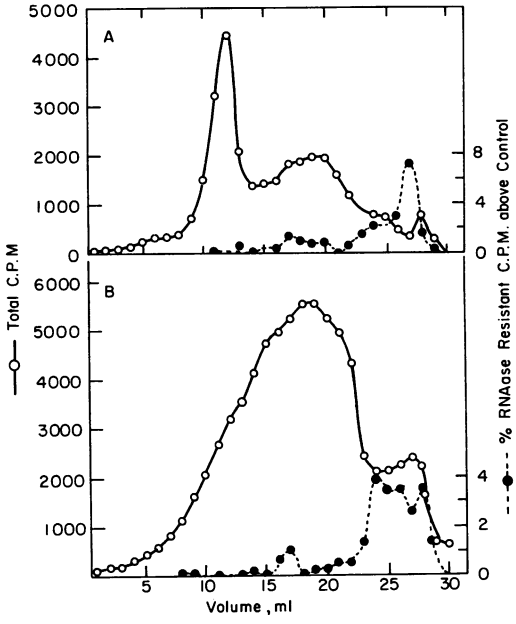

FIG. 4.—Sucrose gradient analysis of P^{32}-TMV- RNA annealed with infected leaf extract. Centrifugation for 16 hr at 24,000 rpm at 4–10°C in SW 25 rotor of model L Spinco, 5–20% sucrose in SSC. (*A*) Sample annealed 12 hr, additional TMV-RNA added prior to centrifugation. RNase-resistant cpm above control refers to the percentage of cpm in each fraction which remain acid-precipitable after treatment with 100 μg/ml RNase (see *Methods*) above the *average* RNase resistance of the entire sample prior to fractionation (2.2%). (*B*) Sample annealed for 6 hr, assayed after centrifugation with 10 μg/ml RNase. The average RNase resistance in this case is 2.9%.

prior to quenching the unlabeled extract. The ability of the extract to confer RNase resistance was completely destroyed on alkaline hydrolysis. Ninety-eight per cent of the RNase resistance of the labeled TMV-RNA could be removed by lowering the salt concentration of the annealed mixture, heating to 100°C, and rapidly cooling the material with an excess of unlabeled TMV-RNA. Furthermore, the ability to confer RNase resistance appeared to be specific for TMV-RNA, since TYMV-RNA of the same specific radioactivity did not acquire appreciable RNase resistance on annealing with the extract.

The irreversible thermal dissociation of RNase resistance of an annealed mixture in 0.01 SSC is shown in Figure 3. There is a sharp transition with a *Tm* of 74°C.

When a constant amount of radioactive TMV-RNA was annealed with varying amounts of extract, it was found that at high TMV-RNA to double-stranded RNA ratios, the amount of hybrid formed varied linearly with the amount of extract used (see Table 2). Using this technique, Weissmann *et al.*[7] have measured the number of "replicative forms" per bacterial cell infected with MS-2 phage. Assuming 50 per cent recovery of the double-stranded RNA from leaves and 100 per cent annealing efficiency, an estimate of the number of double-stranded molecules per infected leaf can be made. In leaves from plants infected 10 days (see *Methods* for details of infection), we find an average of 10^9 such molecules per leaf; 16 days after infection this number rises to 10^{11} molecules per leaf. If in systemically infected leaves all of the 10^8 cells are infected, these numbers correspond to 100 and

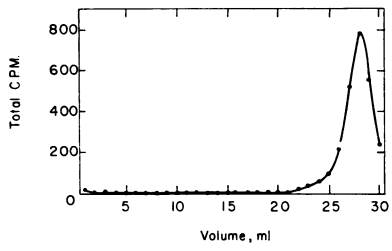

FIG. 5.—Sucrose gradient analysis of P^{32}-TMV-RNA annealed with infected leaf extract for 6 hr and treated with 10 μg/ml RNase for 20 min in 2 SSC at 27°C prior to centrifugation. Conditions of centrifugation as in Fig. 4.

1000 double-stranded molecules per cell, respectively. For comparison, these cells contain from 10^5 to 10^6 mature virus particles.[17]

Sedimentation analyses of the annealed mixtures were carried out on sucrose gradients. Figure 4*A* shows the sedimentation pattern of such a mixture that had TMV-RNA added just before centrifugation to serve as a marker. The 30s viral RNA is followed by a broad band of viral RNA that has been degraded during the annealing process. When the RNase resistance of each fraction was measured after centrifugation, most of the remaining radioactivity appeared in low *S* regions of the gradient.[10] This material had sedimentation coefficients of 4s–10s. There is a suggestion, however, of a peak of activity in the 15s–20s region. Figure 4*B* shows a similar gradient of material that has been annealed half as long as the above material. Again, the RNase-resistant material is very heterogeneous and appears in the 4s–10s region, with a small peak in the 15s–20s region of the gradient.

When an annealed mixture is treated with RNase before centrifugation, the result shown in Figure 5 is obtained. Most of the RNAse-resistant material has an *S* of 5 or less. If an aliquot from each fraction of the gradient is again exposed to RNase, enzyme resistance coincides with the observed peak, and 70 per cent of the total radioactivity remains RNase-resistant.

Discussion.—The results presented show that elements capable of conferring RNase resistance upon P^{32}-labeled TMV-RNA exist in nucleic acid extracts from leaves infected with TMV. The following properties of these elements suggest that they are double-stranded RNA: resistance to RNase and DNase, sensitivity to alkaline hydrolysis under conditions that do not degrade DNA, and the requirement for thermal dissociation in low ionic strength media prior to annealing with labeled viral RNA. The sharp thermal transition of susceptibility of RNase of TMV-RNA that has been annealed with these elements indicates that the protection afforded is due to conformational properties of the annealed mixture. Together with the

TABLE 2

DILUTION TEST OF ABILITY OF INFECTED LEAF EXTRACT TO CONFER RNASE RESISTANCE UPON P^{32}-LABELED TMV-RNA

Sample number	Volume of extract, ml	P^{32}-viral RNA	Molar ratio* P^{32}-RNA/TMV-RNA^{-1}	Cpm RNAse-resistant RNA above control	μg P^{32}-RNA annealed	μg TMV-RNA^{-1} per ml extract
1	1.0	TMV	7.4	67,870	28.0	56.0
2	0.5	TMV	14.7	40,940	16.9	67.6
3	0.1	TMV	73.7	7,620	3.15	63.0
4	0.05	TMV	147	4,120	1.70	64.0
5	1.0	TYMV	11	438†	0.01	—
6	0.1	TYMV	22	370†	0.08	—

* Calculated on basis of 63 μg TMV-RNA^{-1} per ml extract.
† Normalized to same specific radioactivity as TMV-RNA.
The volume of extract indicated plus sufficient 0.01 SSC to make a final volume of 1 ml was heated to 100°C for 10 min in a sealed tube. 231 μg of TMV-RNA with specific activity 2,420 cpm/μg was added at 100°C. Sufficient 10 SSC was added to make the solution 2 SSC. Annealing and assays were performed as described in *Methods*. One milliliter of extract contained the nuclease-resistant material from 210 leaves infected 16 days. The control, consisting of viral RNA carried through the heating and annealing process without extract, and assayed as above, gave 15,190 cpm remaining acid-precipitable. The extract has an absorbancy of 80 at 260 mμ.

dilution test (Table 2), these facts suggest that the elements contain a strand of viral RNA and another strand at least part of which is complementary to viral RNA. Finally, the lack of protection afforded P^{32}-RNA from another plant virus confirms the specificity of the interaction.

We have no evidence that the double-stranded elements observed are obligatory intermediates, or even intermediates, in the replication of TMV. It seems worth while to consider the possibility that the real intermediate in viral RNA replication is a labile form containing a complementary RNA strand, and that the double-stranded forms isolated are products of rearrangements *in vitro* or *in vivo* by mechanisms which are not in the direct path of virus biosynthesis. In view of the very small number of leaf cells that can be infected initially, and current limitations on the extent of labeling of plant viruses, it is not likely that this question can be decided with plant-virus leaf systems.

Summary.—Tobacco leaves systemically infected with TMV contain elements which are nuclease-resistant, alkali-labile, and are capable, after suitable dissociation and annealing, of conferring ribonuclease resistance upon P^{32}-labeled TMV-RNA. The latter property is specific for TMV-RNA, and identifies the protective elements as RNA strands containing nucleotide sequences complementary to those in TMV-RNA, and which prior to dissociation were contained in double-stranded forms. The infected cells studied contain from 10^2 to 10^3 viral equivalents of double-stranded RNA, or one per 10^3 mature virus particles.

We should like to thank F. Fox and S. B. Weiss for a gift of RNA polymerase, and E. P. Geiduschek for a critical reading of the manuscript.

* This study was supported by grants AI-04448-03, GRS 1-SO 1-FR-05367-01, and graduate training grant 1 T1 AI-238 from the USPHS.

† Predoctoral trainee of the USPHS, grant no. 2 T1 GM 780.

‡ Research Career Development Awardee of the USPHS.

[1] Sinsheimer, R. L., B. Starman, C. Nagler, and S. Guthrie, *J. Mol. Biol.*, **4**, 142 (1962).

[2] Geiduschek, E. P., J. W. Moohr, and S. B. Weiss, these PROCEEDINGS, **48**, 1078 (1962).

[3] The following abbreviations have been used: TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus; EMC, encephalomyocarditis; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; EDTA, ethylene diamine tetracetic acid; A, G, U, C, adenylic, guanylic, uridylic, and cytidylic acids, respectively; SSC, 0.15 *M* NaCl, 0.015 *M* Na_3 citrate, pH 7.1; 0.01 SSC, 2 SSC, and 10 SSC are multiples of the same salt solution; RNase, pancreatic ribonuclease; DNase, pancreatic deoxyribonuclease; TMV-RNA^{-1}, hypothetical strand of RNA complementary to TMV-RNA; C-RNA, complementary RNA, the double-stranded product of RNA polymerase *in vitro*.

[4] Montagnier, L., and K. Sanders, *Nature*, **199**, 664 (1963).

[5] Baltimore, D., Y. Becker, and J. Darnell, *Science*, **143**, 1034 (1964).

[6] Kelly, R. B., and R. L. Sinsheimer, *J. Mol. Biol.*, **8**, 602 (1964).

[7] Weissman, C., P. Borst, R. H. Burdon, M. A. Billeter, and S. Ochoa, these PROCEEDINGS, **51**, 682 (1964).

[8] Kaerner, H. C., and H. Hoffmann-Berling, *Nature*, **202**, 1012 (1964).

[9] Fenwick, M. L., R. L. Erikson, and R. M. Franklin, *Federation Proc.*, **23**, 319 (1964).

[10] Tobey, R. A., *Virology*, **23**, 10 (1964).

[11] Hoagland, D. R., and D. Arnon, *Calif. Univ. Agr. Expt. Sta. Circ.*, No. 347 (1950).

[12] Boedtker, H., and N. Simmons, *J. Am. Chem. Soc.*, **80**, 2550 (1958).

[13] Fraenkel-Conrat, H., B. Singer, and A. Tsugita, *Virology*, **14**, 54 (1961).

[14] Haselkorn, R., V. A. Fried, and J. E. Dahlberg, these PROCEEDINGS, **49**, 511 (1963).

[15] Fox, C. F., W. S. Robinson, R. Haselkorn, and S. B. Weiss, *J. Biol. Chem.*, **239**, 186 (1964).

[16] Nakamoto, T., C. F. Fox and S. B. Weiss, *J. Biol. Chem.*, **239**, 167 (1964).

[17] Wildman, S. G., in *The Viruses*, ed. F. M. Burnet and W. M. Stanley (New York: Academic Press, 1957), vol. II, p. 3.

---

*THE RESPONSE OF THREE CATALYTICALLY FUNCTIONAL GROUPS IN CHYMOTRYPSIN TO CHANGES IN ACIDITY**

BY WM. F. SAGER AND PAUL C. PARKS

CHEMISTRY DEPARTMENT, THE GEORGE WASHINGTON UNIVERSITY

Communicated by George S. Hammond, June 30, 1964

Of the various techniques for investigation of enzyme reaction mechanisms, few have received less attention or systematic study than linear free-energy relationships. Scattered references may be found for a few determinations of Hammett rho constants and there is a voluminous literature describing the influence of structure on rate. The latter studies have dealt with rather gross changes which, though difficult to interpret, nevertheless show that the Taft equation does not hold in many instances. In order to take full advantage of linear free-energy relationships as a probe to identify the nature of the enzymatic process, two considerations are paramount. Substituent changes should be made at points sufficiently distant from the reaction site to ensure good linear free-energy relations in nonenzymatic reactions, and the enzymatic process should be studied in sufficient detail to ensure that a linear free-energy relationship, if it does exist, is not overlooked due to complications brought about through competing processes or complex changes due to acidity, inhibition, and so forth.

This publication is the second in a series dealing with a systematic study of the scope and application of Hammett relationships to the study of enzyme mechanisms. In the first we have shown the necessity for protonation of the amide nitrogen in amide hydrolysis, a fact which is in no way astonishing but which illustrates one specific application of the method.[1] In this, the second, we will show how by application of the Hammett equation it is possible to distinguish between the influence of acidity on the ground state and on transition state, a feature of utmost importance if the identities of the catalytically influential groups are to be identified through investigations of the pH-rate profile.

It was previously found that the observed rate constants for alpha-chymotrypsin-catalyzed hydrolysis of a series of N-benzoyl-L-tyrosine substituted anilides follow the Hammett equation with a negative value of rho at pH 6.92.[1] These studies have been extended through the pH range[2] 5.585 to 10.022 at 0.212 pH-unit intervals for the above anilides with substituents m-CH_3O-, H-, and p-CH_3O-. The method was the same as that previously reported. The solvent used was 1:3 v/v dimethylsulfoxide-water, which was 0.1 *M* in KCl and 0.0001 *M* in $CaCl_2$. The temperature was 25.18–0.02°C. These reactions obey pseudo first-order kinetics to 98 per cent completion at all pH values studied.

If considered alone, the knowledge to be gained from pH dependence studies is limited. The pH dependence of the free-energy difference between ground and