Reduced Acid-Neutralizing Velocity of Spray-Dried Agglomerated Magnesium Carbonate

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Abstract □ The rate of neutralization of acid by spray-dried products bound with gum arabic, gelatin, polyvinyl alcohol, carboxymethylcellulose, methylcellulose, and polyvinylpyrrolidone was investigated to evaluate antacid and timed-release properties. A double-layer model was assumed to analyze the neutralization velocity. Diffusion and the reaction layers at the interface between solid and liquid were considered, and an acid neutralization rate equation was developed. The mass transfer coefficient and reaction rate constant for acid neutralization were determined to be 9.3 $\times 10^{-3}$–7.0 $\times 10^{-2}$ cm sec^{-1} and 400–500 cm^3 sec^{-1}, respectively. The percentage of spray-dried particles coated with binder was 80% for carboxymethylcellulose and 48–65% for gelatin, methylcellulose, and polyvinyl alcohol products. Gum arabic and polyvinylpyrrolidone products proved to be only agglomerated. Tableting the powdered spray-dried products increased the proportion of encapsulated material from 48–83 to 70–98%, and their timed-release capacity was greater than that prior to tableting.

Keyphrases □ Magnesium carbonate (spray-dried agglomerated)—reduced acid-neutralizing velocity, model, equations □ Acid-neutralizing velocity—reduced rate of spray-dried agglomerated magnesium carbonate, model, equations □ Neutralization rate of antacids—spray-dried products bound with gum arabic, gelatin, polyvinyl alcohol, carboxymethylcellulose, methylcellulose, and polyvinylpyrrolidone, model, equations □ Antacids—spray drying, effect on acid-neutralizing velocity □ Spray drying—magnesium carbonate, effect on acid-neutralizing velocity, model, equations

Spray drying has been widely applied in the pharmaceutical industry (1–3). One interesting application is as a microagglomeration and encapsulation technique for the preparation of timed-release dosage forms. Kornblum (4) prepared a sustained-action tablet using spray-dried powders, while Kawashima *et al.* (5) produced microencapsulated agglomerates by spray drying.

Magnesium carbonate is frequently used as an antacid and laxative, and many studies have evaluated its antacid capacity (6–8) and neutralizing velocity (9, 10). However, little is known about the neutralizing velocity of spray-dried agglomerated magnesium carbonate. The present work was undertaken to evaluate the antacid properties of the spray-dried material as a component of timed-release medicaments. Neutralizing velocity was analyzed by a double-layer model, which yielded separately the rate constants for diffusion and for reaction. The proportion of encapsulated product was also determined to allow various binders to be evaluated for effectiveness in producing timed-release behavior.

EXPERIMENTAL

Test Samples—Test antacids were unagglomerated magnesium carbonate, having a diameter of 2–8 μm and specific surface area by the nitrogen adsorption method of about 20 m^2 g^{-1}, and spray-dried agglomerated products bound with gum arabic, gelatin, polyvinyl alcohol, carboxymethylcellulose, methylcellulose, or polyvinylpyrrolidone. The binders were used at a level of 0.5–3% by weight of solids, and the aqueous slurries were atomized centrifugally and dried at 150 ± 10°. Details of the technique may be found in Ref. 11. Representative particle properties of the spray-dried products were as follows: volume surface mean diameter, 26–78 μm; true particle density, 2.1–2.3 g cm^{-3}; and specific surface area by adsorption, 13–20 m^2 g^{-1}.

Five samples of salicylic acid, having diameters of 4, 30, 58, 89, or 150 μm and a true density of 1.4 g cm^{-3}, were also used for dissolution rate studies.

Apparatus and Procedure—*Dispersed Powder in Beaker Method*—Powdered antacids (0.5 g) were added to 0.01 *N* HCl (450 ml) in the standard vessel with baffles (Fig. 1A) and maintained at 27, 37, or 47° in a water bath. A dispersed system was produced by constant agitation at 630, 1100, or 1640 rpm using a four-blade stirrer connected with a synchronous motor. At lower speeds, a considerable proportion of particles would float on the surface of the liquid. Acid neutralization was followed by measuring the pH of the solution at adequate intervals using a pH meter with calomel and glass electrodes.

The dissolution of salicylic acid particles into distilled water (450 ml) was investigated under the same experimental conditions. At suitable intervals, 1 ml of solution was withdrawn by pipet. After passage through a glass wool filter, the sample was diluted appropriately with distilled water and the salicylic acid content was determined spectrophotometrically at 298 nm. When powders smaller than 20 μm were used, a small amount of polyoxyethylene sorbitan monooleate was added to the acid solution to prevent agglomeration.

Rotating-Disk Method—Disks of 2.0 g and 2.0-cm diameter were prepared by compression of powdered antacids or salicylic acid at 50 kg cm^{-2} in a compression punch and die. Each disk was mounted on a six-blade agitator of 4.7-cm diameter with a suitable water-insoluble adhesive so that only one face remained exposed (Fig. 1B). The agitator was then immersed in bulk liquid at 27, 37, or 47° and rotated at 150, 241, 382, or 620 rpm. Analytical procedures were the same as for the beaker method.

Measurement of Diffusion Coefficients—Diffusion coefficients were measured by a diaphragm method (12, 13) using four cells with two chambers separated by a diaphragm. Cell constants were determined to be 6.37–9.70 using 0.1 *N* KCl, which is known to have diffusion coefficients of 1.68, 1.78, and 2.28 $\times 10^{-5}$ cm^2 sec^{-1} at 20, 25, and 37°, respectively. Diffusion coefficients of 2 ×

Table I—Diffusion Coefficients (centimeters^2 second^{-1})

	Temperature			
	27°	37°	47°	Literature Data
Salicylic acid[a]	4.10×10^{-6}	4.85×10^{-6}	5.75×10^{-6}	4.2×10^{-6} [b]
Hydrochloric acid[a]	2.31×10^{-5}	2.52×10^{-5}	2.79×10^{-5}	$2.38 \pm 0.04 \times 10^{-5}$ [c]

[a] Concentration of salicylic acid and hydrochloric acid is 2×10^{-3} mole liter^{-1} and 0.01 *N*, respectively. [b] Diffusion coefficient at pH 3 and 37°, Ref. 18. [c] Diffusion coefficient at 12°, Ref. 19.

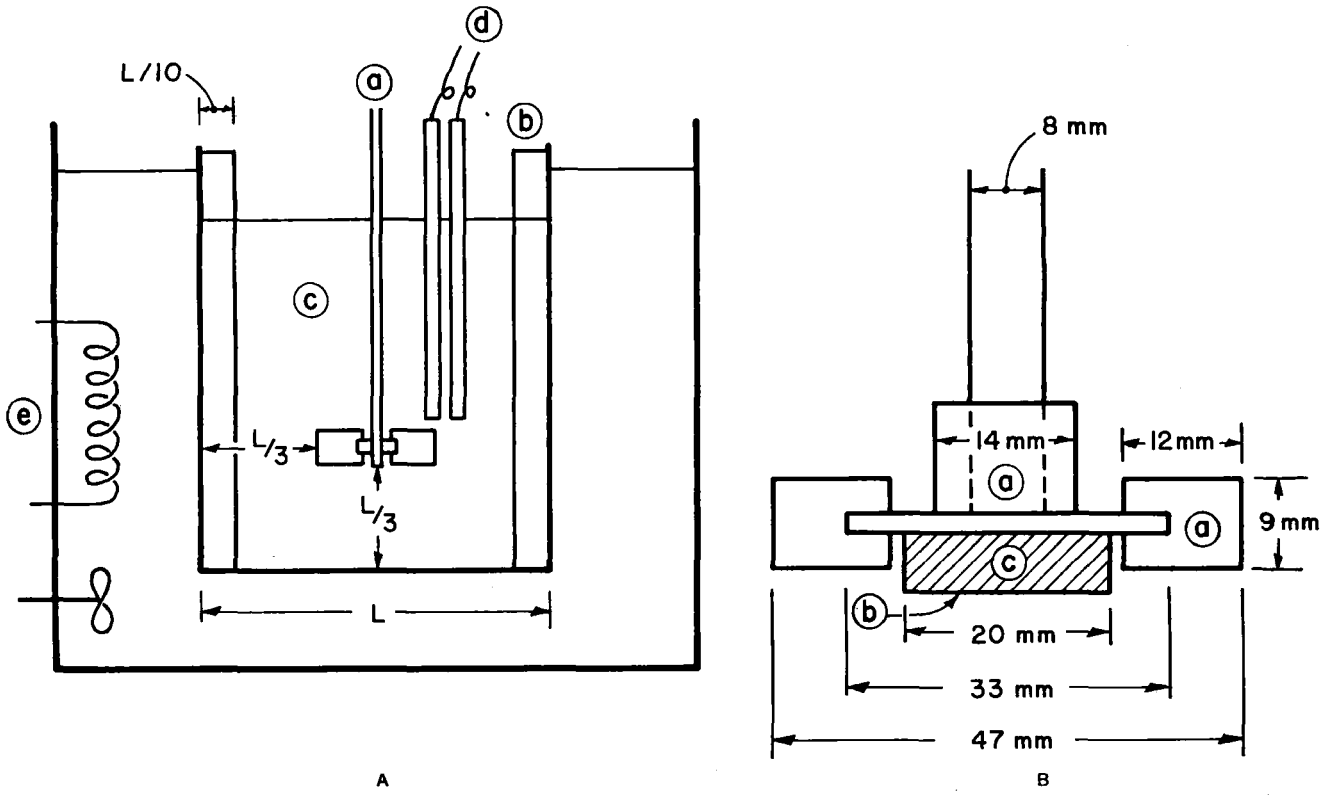

Figure 1—*Apparatus and procedure for measuring pH changes in dispersed system and rotating-disk method. (A) Dispersed system consisting of the standard vessel of 87 mm diameter. Key: a, stirrer with four blades; b, baffle; c, acid solution of 450 ml; d, pH meter; and e, water bath with thermal unit. (B) Rotating disk. Key: a, impeller with six blades; b, tablet surface exposed to acid solution; and c, adhesives.*

10^{-3} mole liter^{-1} salicylic acid and 0.01 *N* HCl were determined at 27, 37, and 47° (Table I).

Mechanism and Rate Equation of Acid Neutralization—In this work, the rate equation for acid neutralization was developed by considering both hydrogen-ion transfer and the neutralizing reaction at the interface between solid and bulk liquid. By assuming the presence of a reaction layer and a diffusion layer, a model for acid neutralization was introduced (Fig. 2).

Hydrogen ions diffuse through the diffusion layer into the reaction layer deposited on the solid surface and the neutralization reaction occurs in the reaction layer. Two assumptions were made:

1. The hydroxyl ions produced by dissolution of the magnesium carbonate reach the reaction layer at a much higher rate than the hydrogen ions coming through the diffusion layer.
2. A steady concentration of hydrogen ions in the reaction layer is established, and the net rate of change of hydrogen-ion concentration there is set equal to zero.

When using these assumptions, Eqs. 1 and 2 may be derived:

$$-\frac{d}{dt}(H_1) = \frac{k_1 A}{V}(H_1 - H_2) \qquad \text{(Eq. 1)}$$

$$-\frac{d}{dt}(H_2) = \frac{k_1 A}{\Delta v}(H_1 - H_2) - \frac{k_2}{\Delta v} H_2^{\alpha} = 0 \qquad \text{(Eq. 2)}$$

where:

A = surface area of antacid (centimeters^2)
k_1 = mass transfer coefficient (centimeters second^{-1})
k_2 = reaction rate constant (centimeters^3 second^{-1})
H_1 = hydrogen-ion concentration in bulk liquid (moles liter^{-1})
H_2 = hydrogen-ion concentration in reaction layer (moles liter^{-1})
t = time
V = volume of bulk liquid (centimeters^3)
Δv = volume of reaction layer (centimeters^3)
α = reaction order

The rate of reduction of hydrogen ions in the bulk liquid is given by:

$$-\frac{d}{dt}(H_1) = \frac{k_2}{V} H_2^{\alpha} \qquad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

Determination of Mass Transfer Coefficients and Reaction Rate Constants—If the neutralization reaction is first order with respect to hydrogen-ion concentration in the reaction layer (*i.e.*, $\alpha = 1$), Eq. 1 becomes:

$$-\frac{d}{dt}(H_1) = \frac{k_1 k_2 A}{V(k_1 A + k_2)} H_1 \qquad \text{(Eq. 4)}$$

Equation 4 may be integrated to yield Eq. 5, considering the initial condition $H = H_0$ or pH = pH_0 = 2.0 at $t = 0$:

$$-\ln H_1 = \frac{k_1 k_2 A}{V(k_1 A + k_2)} t - \ln H_0 \qquad \text{(Eq. 5)}$$

Equation 5 is transformed into a more convenient form:

$$pH - pH_0 = \frac{k_1 k_2 A}{2.303 V(k_1 A + k_2)} t \qquad \text{(Eq. 6)}$$

Acid neutralization of powdered unagglomerated magnesium carbonate was carried out in the dispersed system. Plots of the acidity change, pH − pH_0, are shown in Fig. 3. For clarity, only the data points taken at 10-sec intervals are shown, although readings were made every 5 sec in the early stages where pH was changing rapidly. After short induction periods of about 3–7 sec, linear relationships are obtained as required in Eq. 6. Their slopes, tan α, are represented as:

$$\tan\alpha = \frac{k_1 k_2 A}{2.303 V(k_1 A + k_2)} \qquad \text{(Eq. 7)}$$

The measured values of the slope are given in Table II where it

Table II—Mass Transfer Coefficients and Reaction Rate Constants for Acid Neutralization

Experimental Condition	tan α[a]	Average	Average Deviation, %	$X \times 10^{3}$[a]	$D \times 10^{5}$[a]	$k_1 \times 10^{2}$[a]	$k_2 \times 10^{-2}$[a,b]
37°, 630 rpm[a]	0.317 0.302 0.296	0.305 ± 0.009	2.6	2.30	2.52	1.10	
37°, 1100 rpm	0.320 0.326 0.314	0.320 ± 0.006	1.3	0.830	2.52	3.04	4.14
37°, 1640 rpm	0.390 0.378 0.387	0.385 ± 0.006	1.2	0.400	2.52	6.30	
27°							4.05
47°							4.52

[a] tan α = slope of pH *versus* time straight line (pH units second^{-1}); X = thickness of diffusion layer (centimeters); D = diffusion coefficient of hydrochloric acid (centimeters^2 second^{-1}); k_1 = mass transfer coefficient (centimeters second^{-1}); k_2 = reaction rate constant (centimeters^3 second^{-1}); rpm = agitation speed. [b] The reaction rate constant is the average of three experimental results at 630, 1100, and 1640 rpm.

is seen that these values were reproducible to within an average deviation of ±3% or less. As the reaction proceeds, the surface area of antacid decreases and the lines become curved. It is impossible to determine simultaneously both the mass transfer coefficient and the reaction rate constant from Eq. 7, so one of these constants must be estimated by another method (14).

Double-film theory (15) represents the mass transfer coefficient as:

$$K = \frac{D}{X} \qquad \text{(Eq. 8)}$$

where D = diffusion coefficient (centimeters^2 second^{-1}), K = mass transfer coefficient (centimeters second^{-1}), and X = thickness of diffusion layer (centimeters). The mass transfer coefficient for the hydrogen ion can be determined from Eq. 8 when the diffusion coefficient of hydrochloric acid and the thickness of the diffusion layer at the interface between antacid and bulk liquid are measured. Jost (16) expressed the diffusion layer thickness in the case of a solvent flowing linearly over a dissolving solid by:

$$X = \sqrt{\frac{\eta\xi}{d\gamma}} \qquad \text{(Eq. 9)}$$

where η = viscosity of the fluid (poises), ξ = linear dimension of the surface of the solid across which the dissolution medium flows (centimeters), γ = velocity of flow (centimeters second^{-1}), and d = density of the medium.

It is difficult to measure ξ in Eq. 9. In this study the thickness of the diffusion layer was estimated by analyzing the rate of powdered salicylic acid dissolution under the same experimental conditions (fluid properties, particle size, agitation, *etc.*) as those used in the acid neutralization study. The Hixson and Crowell (17) cube-root law for dissolution rate was rearranged to give:

$$\frac{d}{dt}\left(\frac{C_L}{C_e}\right) = \frac{DS_wC_e}{X}\left(1 - \frac{C_L}{C_e}\right)^{5/3} \qquad \text{(Eq. 10)}$$

where:

C_L = concentration of solute (moles liter^{-1})
C_e = equilibrium concentration of solute at $t = \infty$ (moles liter^{-1})
S_w = specific surface area given by $6/(\rho D_0)$ (centimeters^2 gram^{-1})
D_0 = mean particle diameter (centimeters)
ρ = true density of particles (grams centimeter^{-3})

The results of the dissolution experiments satisfied Eq. 10, yielding straight lines with slopes of 1.66 for log-log plots of $(d/dt)\ (C_L/C_e)$ against $[1 - (C_L/C_e)]$. The thickness of the diffusion layer was obtained from:

$$X = \frac{DS_wC_e}{(d/dt)(C_L/C_e)_{t=0}} \qquad \text{(Eq. 11)}$$

The relationships between diffusion layer thickness and the experimental conditions are shown in Fig. 4 for both the dispersed and the rotating-disk systems. As might be expected from Eq. 9, the thickness of the diffusion layer decreased with increasing temperature and agitator speed and increased with increasing particle size. Mass transfer coefficients of hydrogen ion were then determined by substituting the diffusion layer thickness, estimated from Fig. 4, and the diffusion coefficient for hydrochloric acid into Eq. 8. Subsequently, the reaction rate constants for acid neutralization were calculated from Eq. 7, using the initial slopes of the plots in Fig. 3. The mass transfer coefficients and the reaction rate constants for acid neutralization are given in Table II.

Determination of Encapsulation Ratio of Spray-Dried Products—A previous study (5), using a scanning electron microscope, showed that spray-dried products are not only agglomerated but that the surfaces of the individual particles in the agglomerates are coated with binder in varying degrees. The proportion of coated surface affects the rate of acid neutralization, and the effective surface area for reaction may be interpreted in terms of an "encapsulation ratio." The effects of spray drying are shown in Fig. 5, where it is seen that the neutralization rates are reduced

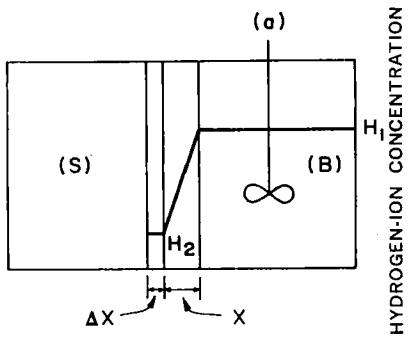

Figure 2—*Double-layer model for acid neutralization. Key: a, stirrer; B, bulk liquid; S, solid; and X and ΔX, diffusion and reaction layer.*

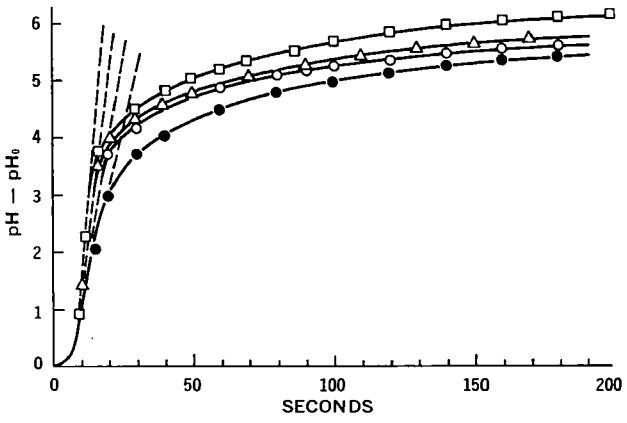

Figure 3—*The pH change* versus *time curves for unagglomerated magnesium carbonate powder at 37°. Key: □, 1640 rpm; △, 1100 rpm; ○, 620 rpm; and ●, 391 rpm.*

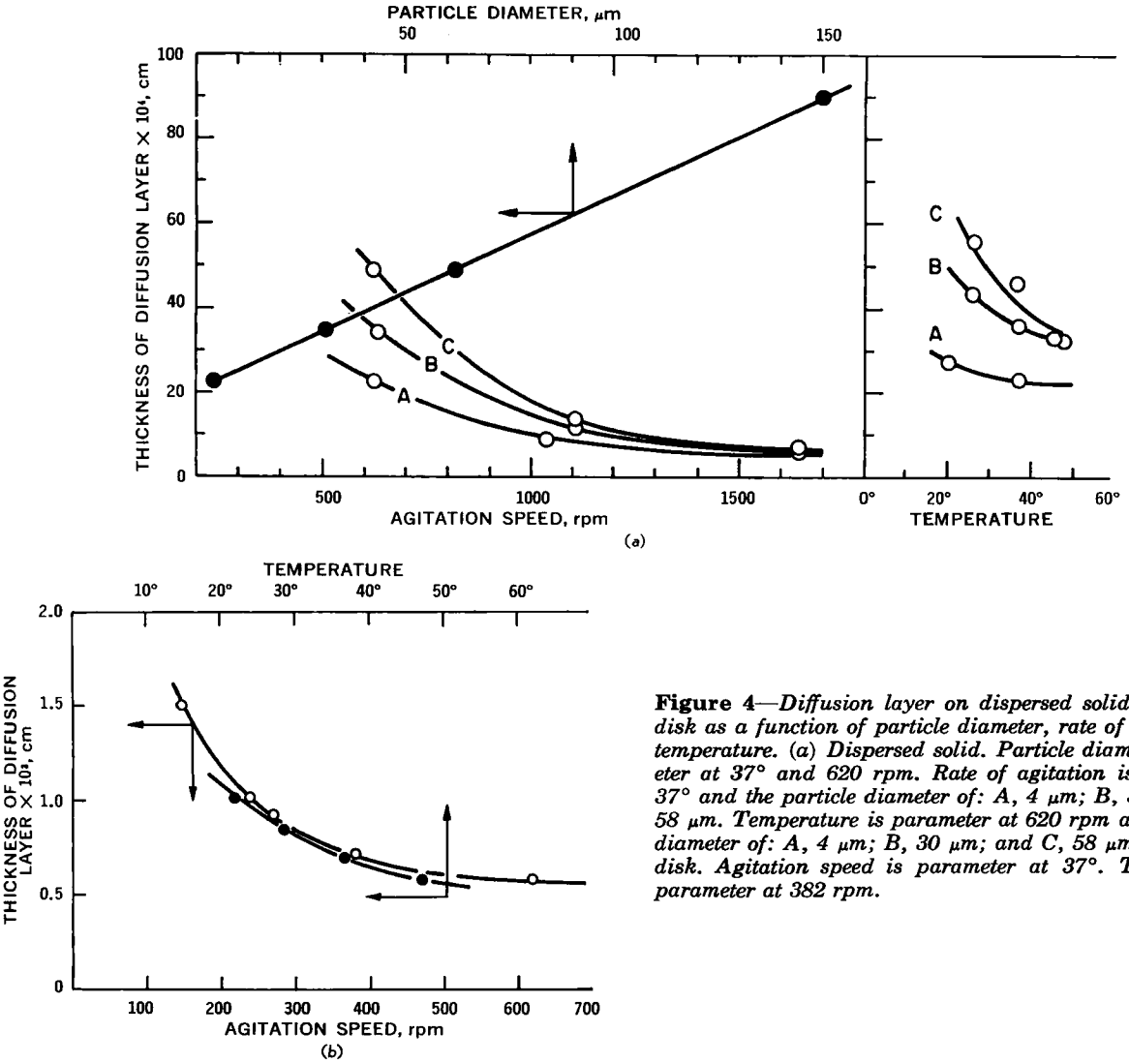

Figure 4—*Diffusion layer on dispersed solid and rotating-disk as a function of particle diameter, rate of agitation, and temperature. (a) Dispersed solid. Particle diameter is parameter at 37° and 620 rpm. Rate of agitation is parameter at 37° and the particle diameter of: A, 4 μm; B, 30 μm; and C, 58 μm. Temperature is parameter at 620 rpm and the particle diameter of: A, 4 μm; B, 30 μm; and C, 58 μm. (b) Rotating disk. Agitation speed is parameter at 37°. Temperature is parameter at 382 rpm.*

compared to those from the untreated magnesium carbonate. In Table III, the encapsulation ratios for the various products are given. The encapsulation ratio is defined by:

$$C_r = \left(1 - \frac{A_c}{A_0}\right) \times 100 \qquad \text{(Eq. 12)}$$

where A_c = effective surface area of particles for acid neutralization (centimeters^2), A_0 = surface area of particles measured by adsorption method (centimeters^2), and C_r = proportion of coated particles (percent).

The effective surface area was calculated by substituting the mass transfer coefficient estimated using Fig. 4 and Table I, the reaction rate constant listed in Table II, and the slope of pH change *versus* time straight line in Fig. 5 into:

$$A_c = \frac{2.303V \tan \alpha k_2}{k_1(k_2 - 2.303V \tan \alpha)} \qquad \text{(Eq. 13)}$$

Table III*a* shows that encapsulation was most effective with carboxymethylcellulose, gelatin, methylcellulose, and polyvinyl alcohol while gum arabic and polyvinylpyrrolidone products appeared to be uncoated; that is, they were only agglomerated.

The acid neutralization of tableted spray-dried products was also investigated using the rotating-disk method. The pH change *versus* time curves by this method at 37° and 382 rpm are shown in Fig. 6. As expected, the rate of pH change for the tablet was much slower than for the powder. Tablets of unagglomerated magnesium carbonate and polyvinylpyrrolidone products showed a similar pH profile. Disintegration of both tablets occurred after 1.5–~2.0 min of residence time. After the disintegration, pH change *versus* time curves were similar to those for the dispersed powder system. Acid neutralization velocities for gelatin and gum arabic products were slower than for unagglomerated and polyvinylpyrrolidone products. Methylcellulose and carboxymethylcellulose products showed a characteristic timed-release action and did not neutralize the acid completely after a day. These tablets did not disintegrate, and

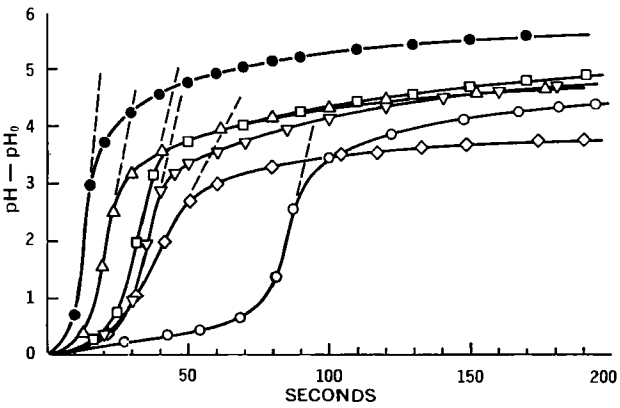

Figure 5—*The pH change versus time curves for powdered spray-dried magnesium carbonate. Key: ●, unagglomerated; △, gelatin; □, gum arabic; ◇, carboxymethylcellulose; ▽, polyvinyl alcohol; and ○, methylcellulose.*

Table III—Encapsulation Ratio of Spray-Dried Products of Magnesium Carbonate

Sample	Experimental Condition	$S_w \times 10^{-4}$ [a]	$A_0 \times 10^{-4}$ [a]	$A_c \times 10^{-4}$ [a]	C_{rd} [a]
(a) Encapsulation Ratios for Powdered Products					
Carboxymethylcellulose, 1% solution	27°, 620 rpm	21.1	10.70	1.75	
Carboxymethylcellulose, 1% solution	37°, 630 rpm	21.1	10.55	2.07	av. 82.8
Carboxymethylcellulose, 1% solution	47°, 620 rpm	21.1	10.62	1.66	
Gelatin, 1% solution		13.7	6.82	2.75	64.8
Gelatin, 3% solution		15.2	7.61	1.69	77.7
Gum arabic, 1% solution		18.4	9.23	9.25	0
Gum arabic, 3% solution		17.9	8.90	3.52	60.5 [b]
Methylcellulose, 1% solution		18.1	9.09	4.49	50.6
Polyvinylpyrrolidone, 0.5% solution		16.5	8.27	8.26	0
Polyvinyl alcohol, 0.5% solution		17.2	8.63	4.49	47.9

Sample	Experimental Condition	A_0	A_c	C_{rt} [a]
(b) Encapsulation Ratios for Tableted Products				
Unagglomerated	37°, 150 rpm		28.6	0.0
Unagglomerated	37°, 241 rpm	26.5 av.	29.7	0.0
Unagglomerated	37°, 382 rpm		22.2	0.0
Unagglomerated	27°, 328 rpm		25.5	0.0
Carboxymethylcellulose, 1% solution		26.5	0.604	97.7
Gelatin, 1% solution		26.5	7.10	73.2
Gelatin, 3% solution		26.5	1.48	94.4
Gum arabic, 1% solution		26.5	26.3	0 [c]
Gum arabic, 3% solution		26.5	1.77	93.3
Methylcellulose, 1% solution		26.5	1.50	94.3
Polyvinylpyrrolidone, 0.5% solution		26.5	27.7	0
Polyvinyl alcohol, 0.5% solution		26.5	7.79	70.6

[a] S_w = specific surface area (centimeters² gram^{-1}); A_0 = actual surface area (centimeters²); A_c = effective surface area for acid-neutralizing reaction (centimeters²); C_{rd} and C_{rt} = encapsulation ratio for powdered and tableted magnesium carbonate, respectively (percent). [b] Value is the average of three experiments at 27, 37, and 47° and 620 rpm. [c] Value is the average of three experiments at 150, 241, and 382 rpm and 37°.

their surfaces dissolved uniformly. The actual surface area of the tablet exposed to the acid solution was found to be 26.5 cm² by substituting experimental results of acid neutralization of unagglomerated magnesium tablet at various temperatures and agitations into Eq. 13. Encapsulation ratios for various kinds of tableted spray-dried products were determined in the same way as for powdered products and resulted in 70–98% (Table III*b*). Comparison of the encapsulation percent of tablets with that of powders (Table III) showed a distinct increase in the encapsulation percent after tableting, especially for the methylcellulose product.

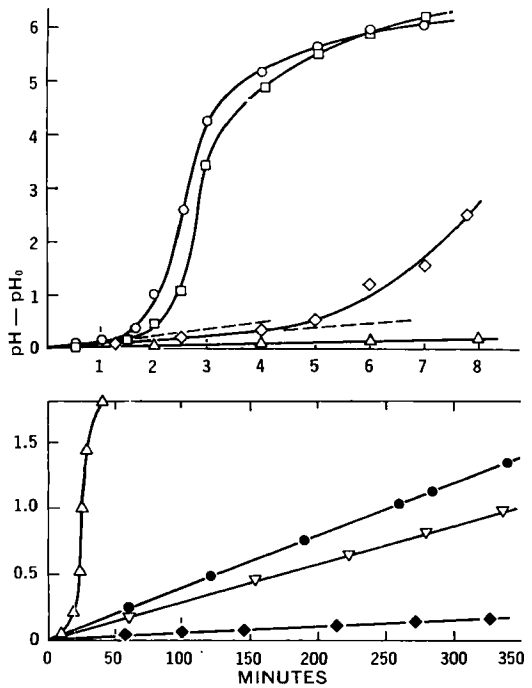

Figure 6—*The pH change versus time curves for tableted spray-dried magnesium carbonate. Key:* □, *unagglomerated;* △, *gelatin;* ▽, *methylcellulose;* ○, *polyvinylpyrrolidone;* ●, *polyvinyl alcohol;* ◇, *gum arabic; and* ◆, *carboxymethylcellulose.*

CONCLUSIONS

Timed-release behavior may be imparted to fine powders by the use of spray-drying agglomeration. In this study, using magnesium carbonate as a model system together with various binders, carboxymethylcellulose, methylcellulose, gelatin, and polyvinyl alcohol were the most effective in reducing the acid-neutralizing rate of the carbonate. Suitable binders apparently not only cause particle agglomeration but also encapsulate the individual particles.

Tableting of spray-dried products increased timed-release properties even further. Applied compression results in closer particle packing and filling of the pores with crushed particles and binder, leading to slower release of the reactive ingredient. Medicaments with release rates arbitrarily controlled over a wide range may thus be prepared using suitable combinations of spray-dried products.

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Excretion of Probenecid and Its Metabolites in Bile and Urine of Rats

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Abstract □ The metabolites of probenecid excreted in the bile of rats with and without ligation of the renal pedicles were investigated using a new GLC assay procedure. Within 8 hr after administration, 63.8% of a 40-mg/kg iv dose of probenecid was accounted for in the bile of normal, anesthetized rats. The metabolites found (as percent of dose) include probenecid (10.0%), probenecid glucuronide (15.7%), glucuronide of the *N*-2-hydroxypropyl derivative (20.3%), glucuronide of the *N*-3-hydroxypropyl derivative (14.2%), and the unconjugated *N*-2-carboxyethyl derivative (3.6%). Ligation of the renal pedicles increased the excretion of each metabolite, raising the total recovery to 86.6%. These oxidized metabolites, but not probenecid or its glucuronide, were excreted in urine in unconjugated form (3–5% each). The unconjugated mono-*N*-depropylated metabolite accounted for 11.2% of the dose in the urine of normal, unanesthetized rats but was not found in the bile of the anesthetized animals.

Keyphrases □ Probenecid—metabolites, excretion in bile and urine of rats with and without renal pedicle ligation, GLC analysis □ Metabolism—probenecid, metabolites excreted in bile and urine of rats with and without renal pedicle ligation, GLC analysis □ GLC—analysis, probenecid metabolites, rats

The metabolic fate of probenecid, which was introduced as a uricosuric agent in 1950, has only recently been elucidated in humans and rats (1, 2). One major metabolite in rat bile was suggested to be the ether glucuronide of *p*-(*N*-propyl-*N*-2-hydroxypropylsulfamoyl)benzoic acid (2). In contrast, a more recent study (3) claimed the major metabolite to be the acyl glucuronide of probenecid. The later conclusion was based on the observation that the peak for probenecid was the only one readily detected by GC of the aglycones released by enzymatic hydrolysis of bile samples from rats given probenecid, 40 mg/kg iv. In the first study (2), the renal pedicles of the animals were ligated; in the later study (3), such ligation was not indicated.

The lack of agreement on the relative amounts of the various metabolites excreted probably arises from the need for a reliable quantitative assay for probenecid and its metabolites in biological fluids. A quantitative procedure based on GLC of the methyl esters for the determination of probenecid and two of its oxidation products is presented. An assay based on the propyl esters, which can be used for all known metabolites of probenecid, will be more completely described in a subsequent publication. These assays were used to measure the excretion of probenecid and its metabolites in the urine and bile of rats and to study the effect of renal ligation on the disposition of the drug and its metabolites in bile.

EXPERIMENTAL

Instrumentation—A gas chromatograph[1], equipped with a flame-ionization detector and a 2.8-mm × 2-m (0.125-in. × 6-ft) stainless steel column packed with 10% OV-1 on 80–100-mesh Chromosorb W-HP, was used. Operating parameters were: column temperature, 225°; injection port, 280°; nitrogen carrier gas flow rate, 23 ml/min; and sensitivity, 2.5×10^{-11} amp full scale.

Procedure—To determine free metabolites, biological samples (1 ml of urine or 0.2 g of bile plus 0.8 ml of water) in 50-ml centrifuge tubes were acidified with 1 ml of 5 *N* HCl and extracted by shaking for 30 min with 20 ml of methylene dichloride. A 10-ml aliquot of the methylene dichloride extract was evaporated to dryness on a water bath (50°) under a stream of air. All subsequent evaporations were conducted in the same way. The residue was dissolved in 0.5 ml of methanol and treated with 2 ml of an ether solution containing approximately 0.3 mmole of diazomethane. The samples were kept at room temperature for 1 hr, after which excess diazomethane was removed by evaporation. The residue was taken up in approximately 2 ml of ether, and a 25–50-μl aliquot containing 75–150 μg of *N,N*-dibenzylbenzenesulfonamide in methanol was added as an internal standard. The ether was evaporated and the residue was taken up in 0.25–0.50 ml of methylene chloride. Approximately a 1-μl aliquot was injected into the gas chromatograph. The quantity of internal standard and the final volume of methylene chloride were chosen so as to keep the GC response within the established range of linearity. Reproducibility was significantly enhanced by making the injection with a 10-μl syringe first loaded with 1 μl of ether adjacent to the plunger and separated from the sample by about 2 μl of air.

[1] Perkin-Elmer Mk II.