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COMPARISON OF THE SPECIFIC CONDUCTIVITIES, BUFFER CAPACITIES AND MOLECULAR WEIGHTS OF FOCUSED AMPHOLINE, SERVALYTE AND PHARMALYTE CARRIER AMPHOLYTES USED IN ISOELECTRIC FOCUSING

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SUMMARY

The specific conductivities of focused Ampholine, Servalyte and Pharmalyte carrier ampholytes have been determined and the results used to establish selection rules for carrier ampholytes in analytical isoelectric focusing.

The buffer capacity of focused Pharmalyte carrier ampholytes has been measured. These data and literature data for the Ampholine and Servalyte systems were used to establish selection rules for carrier ampholytes in preparative isoelectric focusing.

By combining the conductivity data with buffer capacity data for the three carrier ampholyte systems, differences between the molecular weights of these systems were revealed.

INTRODUCTION

Recently, several papers have been published in which the conductivities[1,2] and buffer capacities[3] of focused Ampholine and Servalyte carrier ampholytes were compared. The data were obtained using density gradient[2,3] or gel[1] isoelectric focusing and the results were used to establish selection rules for carrier ampholyte systems. We believe, however, that in establishing such rules a distinction should be made between preparative and analytical applications of isoelectric focusing.

For example, in the comparison of focused Ampholine and Servalyte carrier ampholytes, Fredriksson[3] found the former to have the highest buffer capacity per millilitre (at pH < 8), at identical over-all concentration. Accordingly, he recommended the use of Ampholines. We agree with this recommendation if it is restricted to those applications of isoelectric focusing in which a high buffer capacity of the carrier ampholytes in the focused state is of paramount importance. The rationale for strongly buffering carrier ampholytes lies, of course, in the need for a stable pH gradient, which is not significantly perturbed by the presence of focused proteins. Thus the buffer capacity is the more decisive in the choice of carrier ampholyte, the larger the amount of protein applied. Consequently, in preparative isoelectric focusing the

carrier ampholyte system with the highest buffer capacity per millilitre is preferable, as the prices per millilitre of the two systems are almost equal.

However, in analytical isoelectric focusing, where a high load is without interest, smaller buffer capacities relative to those used in preparative techniques are acceptable and are even preferable, if the resolution is simultaneously enhanced.

In isoelectric focusing the resolution at a certain pH is proportional to the square root of the local field strength and thus (at constant heat production) inversely proportional to the 1/4th power of the local conductivity. Hence, in analytical applications one should rather choose the carrier ampholyte system with the smallest conductivity. Further, it is of interest to use the system with the more even conductivity distribution as a function of pH, as this gives rise to a more even resolution throughout the pH gradient.

Since the appearance of the papers mentioned[1–3], a third type of carrier ampholyte has been introduced by Pharmacia. These so-called Pharmalytes are claimed[4,5] to have such (low and even) conductivities and (excellent) buffer capacities that they provide a superior carrier ampholyte system, unmatched by any other commercially available system. Here also, the distinction between preparative and analytical isoelectric focusing has not been explicitly made.

In many theoretical treatises on isoelectric focusing[6–8] it is stated that (carrier) ampholytes with a high buffer capacity in the isoelectric state also exhibit a high conductivity. However, when two different carrier ampholyte systems are compared, the system with the higher buffer capacity per millilitre at a certain pH will not necessarily also have the higher conductivity in the focused state. This is so because the ratio of these two properties depends on the mean molecular weight of the ampholyte molecules (see Discussion).

Unfortunately, little is known with certainty about the molecular weights of carrier ampholytes. Literature data[9–11] on Ampholines are not consistent. A comparative study[12] of the molecular weights of Ampholines and Servalytes has been published. With respect to Pharmalytes, we have only the statement of the manufacturer at our disposal[4,5].

Therefore, we determined the conductivities of focused Ampholine, Servalyte and Pharmalyte solutions and the buffer capacities of focused Pharmalyte solutions. The focusing was performed in free solution, using the method of Bours[13]. In fact, the interpretation of conductivity measurements on fractions focused in density gradients is difficult owing to the varying solvent composition and to re-mixing during the emptying of the column. With Bours's method both of these difficulties are circumvented and the results are relevant with regard to the choice of the carrier ampholyte system in the most commonly used analytical variant, *viz.*, gel isoelectric focusing.

EXPERIMENTAL

1% (w/v) solutions of Ampholine (LKB, Stockholm, Sweden), pH gradient 3.5–10, Servalyte (Serva, Heidelberg, G.F.R.), pH gradient 2–11, and 40-fold diluted solutions of Pharmalyte (Pharmacia, Uppsala, Sweden), pH gradient 3–10, were focused at 4° in a polyethylene tube (length 150 cm, I.D. 4 mm), coiled on a brass support made according to the description of Bours[13]. The catholyte was 0.067 *M* ethanolamine and the anolyte was 0.017 *M* orthophosphoric acid. After focusing at

a constant voltage (2000 V) for 72 h, the coil was immediately immersed in liquid nitrogen. The tube was cut into 3.5-cm sections, which were allowed to thaw in separate vials. The specific conductivity of these fractions was determined in a cell with cell constant 0.43 cm^{-1} using a precision conductivity meter and frequency generator, types WBR and TAV (Wissenschaftlich-Technische Werkstätten, Weilheim/Obb., G.F.R.). Subsequently the pH was measured as described earlier[14]. For the Pharmalytes the buffer capacity of the fractions was also measured by adding to 250 μl an aliquot of 5 μl of 0.22 *M* hydrochloric acid, followed by titration under nitrogen with 0.24 *M* sodium hydroxide solution and determination of the slope of the titration curve at the original pH value of the fraction. All measurements were performed at 25°. For each carrier ampholyte system duplicate runs were made.

RESULTS

The differences between the conductivity data at identical pH from duplicate runs were random and of the order of 5×10^{-6}–10×10^{-6} Ω^{-1} cm^{-1}, corresponding to a relative difference of 10–20% at the conductivity minimum at $5 < pH < 6.5$ and 0.5–1% at the acidic and basic ends of the pH gradient. The differences between the buffer capacity data for Pharmalytes at identical pH from duplicate runs were random and of the order of 5–10%. Analogous differences were found by Fredriksson[3] in the buffer capacity data from duplicate runs and his explanation also applies here.

In Fig. 1 the buffer capacity for Pharmalytes is plotted against pH, together with data supplied by the manufacturer*. For comparison Fredriksson's data** for

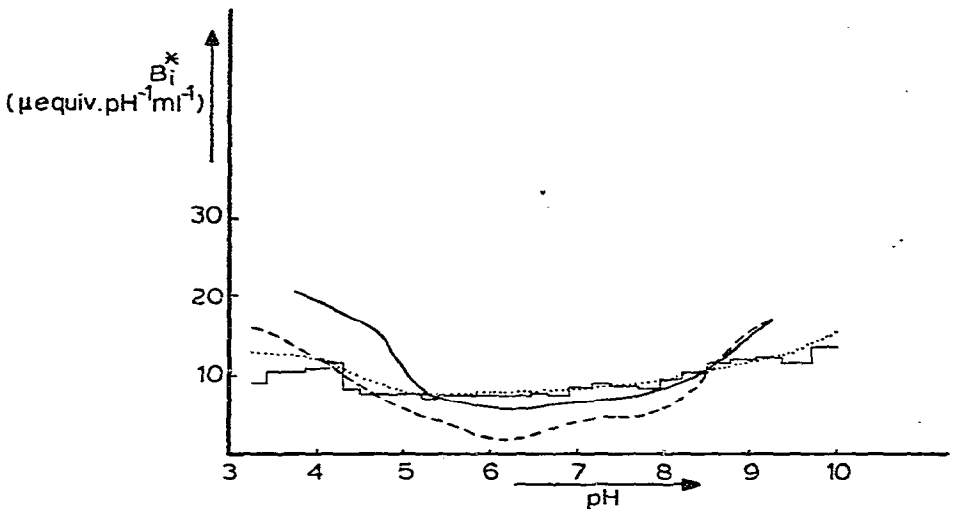

Fig. 1. Buffer capacity *versus* pH of focused Ampholines[3] (———), Servalytes[3] (– – – –), Pharmalytes, this paper (.....) and Pharmalytes[5] (stepwise graph).

* These data are deduced from additional technical information which is available on request[5] (see Discussion).

** A correction to these data for the contribution to the buffer capacity due to sucrose was omitted. Separate determinations of the buffer capacity of sucrose–water mixtures as a function of pH demonstrated that these corrections would amount to about 5, 2 and 3% for Servalytes and 3.5, 1 and 3% for Ampholines at pH 3, 6 and 9, respectively.

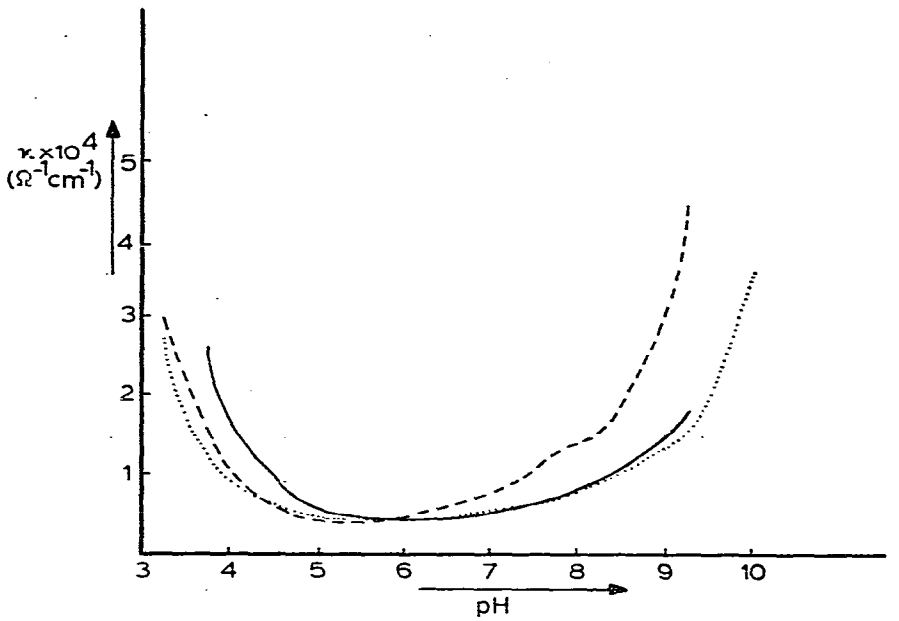

Fig. 2. Specific conductivity *versus* pH of focused Ampholines (———), Servalytes (– – – –) and Pharmalytes (.....).

Ampholines and Servalytes are also given. In Fig. 2 the conductivity is plotted against pH.

DISCUSSION

Our results on the buffer capacity of Pharmalytes are in reasonable agreement with the data supplied by the manufacturer. The stepwise graph in Fig. 1 was deduced from an analogous graph supplied by Pharmacia by dividing the buffer capacity values by 40. Evidently their data refer to the original concentrated solution and appear to have been determined after focusing on Sephadex IEF. Also, the information printed on the bottle (in our case, buffering capacity 0.35 mmole/pH·ml) apparently refers to the original concentrated and unfocused solution.

Comparing the three carrier ampholyte systems (Fig. 1), we observe that Pharmalytes have the largest buffer capacity at $5.5 < \text{pH} < 8.5$ and Ampholines at $\text{pH} < 5.5$, whereas at $\text{pH} > 8.5$ Ampholines and Servalytes have equal buffer capacities, both larger than that of Pharmalytes. Consequently, for preparative isoelectric focusing Pharmalytes are to be preferred at $5.5 < \text{pH} < 8.5$ and Ampholines at $5.5 > \text{pH} > 8.5$ [at $\text{pH} > 8.5$ the smaller conductivity (see Fig. 2) of Ampholines, and thus the better resolution obtainable with this system, favours its use over that of Servalytes].

Our conductivity data in Fig. 2 for Ampholines and Servalytes are in reasonable agreement with those of Fawcett[2] and Righetti *et al.*[1]. The values of Fawcett, obtained by density gradient isoelectric focusing, are lower than ours, as is to be expected from the effect of sucrose. It should be noted that Fawcett did not find

significant differences between the conductivities at identical pH of Ampholines and Servalytes at both ends of the pH gradient, whereas we do. This apparent discrepancy can be explained by the fact that the Servalytes (pH 2–11) cover a wider pH range than the Ampholines (pH 3.5–10). This leads to the situation in the density gradient experiments that at identical basic pH the sucrose concentration in Ampholine fractions is smaller than that in Servalyte fractions, whereas at identical acidic pH the reverse holds.

Although Righetti *et al.* stated that Ampholines and Servalytes display very similar conductivities over the whole pH range, a close inspection of their Fig. 3 reveals conductivity differences at identical pH between the two systems of the same sign as we find. It should be noted, however, that their conductivity values appear to be high in comparison with our values, if a correction with a factor 7 is applied to account roughly for the fact that they used carrier ampholyte concentrations in the gel of 2% but diluted 14-fold in the elution of the gel segments. This may be due to a larger mean degree of dissociation of the ampholytes in their less concentrated solutions, or perhaps to the elution of contaminents from the gel material.

Comparing the three carrier ampholyte systems (Fig. 2), we observe that Pharmalytes combine the favourable properties for analytical applications (*i.e.*, low conductivities) of Servalytes at pH < 6 and of Ampholines at pH > 6. Consequently, in analytical gel isoelectric focusing with narrow pH gradients, the use of Pharmalytes or Servalytes is to be preferred to that of Ampholines at pH < 6, while Pharmalytes or Ampholines should be used instead of Servalytes at pH > 6. With broad pH gradients the use of Pharmalytes is recommended in view of its more even conductivity distribution.

If the influence of sucrose on the mobility and the degree of ionization of carrier ampholyte molecules is identical for the three systems, the same conclusions hold for analytical variants[15–17] of sucrose density gradient isoelectric focusing.

For solutions of model biprotic ampholytes, it has been shown[8] that the molar buffer capacity, B_i, in the isoelectric state is proportional to the degree of ionization, α_i, in the isoelectric state:

$$B_i = (\ln 10)\alpha_i \tag{1}$$

where α_i is defined as $\alpha_i = (c_+ + c_-)/c$. Here $c = c_+ + c_- + c_0$, and c_+, c_- and c_0 represent the concentrations of the positively and negatively charged and neutral species, respectively (mole cm^{-3}). A 1% (w/v) solution will thus have a buffer capacity per millilitre, B_i^*, in the isoelectric state of

$$B_i^* = \frac{\ln 10}{10^2 M} \cdot \alpha_i \tag{2}$$

where M is the molecular weight of the ampholyte. The conductivity contribution, κ_i, of the ampholyte in the isoelectric state is also proportional to the degree of ionization[6]:

$$\kappa_i = F c \bar{u}_i \alpha_i \tag{3}$$

where F is the Faraday constant and $\bar{u}_i$ is the mean of the absolute values of the

mobilities of the charged ampholyte species. For a 1% (w/v) solution, κ_i will thus have the value

$$\kappa_i = \frac{F \bar{u}_i}{10^2 M} \cdot \alpha_i \tag{4}$$

The quotient B_i^*/κ_i thus becomes

$$\frac{B_i^*}{\kappa_i} = \frac{\ln 10}{F \bar{u}_i} \tag{5}$$

As $\bar{u}_i$ can be expected to be inversely proportional to the cube root of the molecular weight, it follows from eqn. 5 that

$$\frac{B_i^*}{\kappa_i} \sim \sqrt[3]{M} \tag{6}$$

We are aware that these expressions do not strictly apply to solutions of the more complicated carrier ampholytes in the focused state, as they are not pure solutions of only one ampholyte species but are contaminated by the adjacent ampholytes. However, in view of the fact that this contamination will influence the buffer capacity and the conductivity in the same (positive) direction, eqn. 6 can be tentatively applied to the results. Therefore, we calculated values of B_i^*/κ_i for the three carrier ampholyte systems.

Values of B_i^* were taken from Fig. 1 and κ_i values were calculated from the conductivity data (κ) in Fig. 2 by subtracting the contribution of the solvent ions (κ_{solv}):

$$\kappa_i = \kappa - \kappa_{solv} \tag{7}$$

κ_{solv} can be shown to be insignificant for pH values around 7 but is equal to

$$\kappa_{solv} = 10^{-3} \Lambda_H \cdot 10^{-pH} \tag{8}$$

for acidic solutions and

$$\kappa_{solv} = 10^{-3} \Lambda_{OH} \cdot 10^{pH-14} \tag{9}$$

for basic solutions. In these equations, κ is expressed in $\Omega^{-1}\,cm^{-1}$ and Λ is the equivalent conductance in $cm^2\,\Omega^{-1}$ equiv.$^{-1}$.

The results are given in Fig. 3, and indicate that the molecular weight of the three carrier ampholyte systems decreases with increasing p*I* over most of the pH gradient. For Ampholines and Servalytes, the production process of which is relatively well known, this effect can be explained if one imagines their synthesis as a reaction of a given polyamine (*viz.*, pentaethylenehexamine) with gradually increasing amounts of an acid (*viz.*, acrylic acid), giving ampholytes with gradually increasing numbers of propionic acid residues per mole and thus gradually decreasing isoelectric points. Moreover, it can safely be assumed that not one but a mixture of polyamines

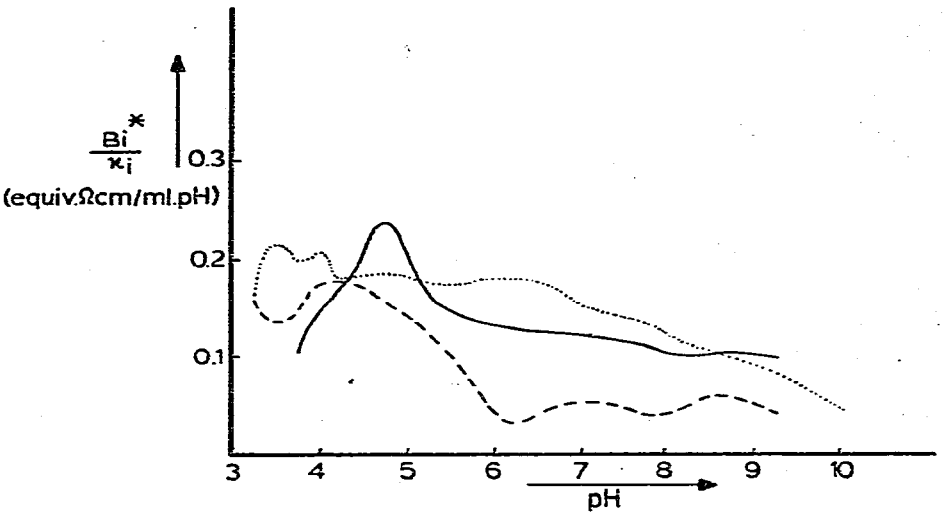

Fig. 3. Values of B_i^*/κ_i *versus* pH of focused Ampholines (———), Servalytes (———) and Pharmalytes (.....).

is used in the synthesis[18–22] and it has been demonstrated both on theoretical[18] and experimental[23] grounds that the higher the molecular weight of the amine, the lower the isoelectric points of the resulting ampholytes generally are.

In this context, the decrease in B_i^*/κ_i with decreasing pH for Ampholines at pH < 4.8 is unexpected. A possible explanation may be that Ampholines of very low isoelectric point, say pH < 4, cannot be obtained in sufficient number with the higher polyamines by the reaction with acrylic acid. In fact, in order to obtain an ampholyte with p$I < 4$, the introduced propionic acid groups must have p$K < 4$. Whereas a decrease in the pK value of propionic carboxyl groups (relative to free propionic acid, pK 4.9) of roughly 1 pH unit has been observed[18] with the higher molecular weight polyaminopolypropionic acids, a decrease of the order of 1.5–2 pH units has actually been found[23] only in small molecules such as ethylenediaminedipropionic acid. The strengthening of the propionic carboxyl groups is due, of course, to the inductive effect of the positive charge on the nitrogen atoms in the ampholyte molecule. It is well known, however, that some of the amine groups in the higher polyamines have extremely low pK values (thus, in triethylenetetramine[18], tetraethylenepentamine[18] and pentaethylenehexamine[24], the lowest pK values are 3.3, 2.7 and 1.1, respectively). Hence a further decrease in the pK value of propionic carboxyl groups in the higher polyaminopolypropionic acids is improbable, as an increasing number of nitrogen atoms are uncharged at pH 4.9.

We think, therefore, that Ampholines with the lower isoelectric points are prepared in separate runs, in which the lower polyamines are used and possibly acetic acid residues are introduced (the inductive effect on the dissociation of acetic carboxyl groups will be stronger), or in which the ampholytes are obtained by introducing some amino groups in suitable dicarboxylic acids. This view is supported by the discussion of Vesterberg[25] on the extension of the pH range of carrier ampholytes on the acidic side, especially by his remark, "much synthesis work had to be done before it was possible to get enough molecules in reasonable yields to cover the pH range

2.5–4". The decrease in B_i^*/κ_i with decreasing pH at low pH values is much less pronounced for the Servalytes. As these contain the much stronger sulphonic acid groups, this is not in contradiction with the above reasoning.

In the comparison of the curves of the three carrier ampholyte systems in Fig. 3, the statements of the manufacturers with respect to the mean molecular weight (Ampholines[9], 600–900; Servalytes[22], 400–700; and Pharmalytes[4,5], 300–600) should be borne in mind. The relative position of the curves for Ampholines and Servalytes agrees qualitatively with these statements. Moreover, it is supported by the observation[2] that Servalytes focus more rapidly than Ampholines and by the results[12] of thin-layer gel chromatography with broad and narrow pH range Ampholines and Servalytes on Bio-Gel P-4. The relatively high molecular weights of Pharmalytes, as indicated in Fig. 3, are in contrast with the above statements. We have no explanation for this discrepancy. It should be noted, however, that the evidence given by the manufacturer[5] (in the form of an elution profile of Pharmalytes of unknown pH range on a mixed gel bed of Sephadex G-15 and G-50) is not convincing, as a molecular weight calibration is lacking. In a recent leaflet[26] from the manufacturer, the hypothetical structure of a "typical" constituent Pharmalyte is given. Substitution of the five R-groups in this structure by the smallest possible substituent (*viz.*, methylamino) gives a molecular weight of 763.

As argued above, the conclusions from Fig. 3 should be regarded as tentative; they certainly need independent experimental confirmation. An argument in favour of the applicability of eqn. 5 to the results is provided by the fact that the mean value of B_i^*/κ_i for Ampholines (0.13 equiv. Ω cm ml^{-1} pH^{-1}) gives a mean molecular weight ($\bar{M}$) of Ampholines of the correct order of magnitude. This can be shown by substituting $\bar{u}_i$ in eqn. 5 by the empirical relation of Edward and Waldron-Edward[27] valid for large monovalent organic ions ($r_w > 2.7$ Å):

$$u^0 = \frac{1.14 \cdot 10^{-3}}{r_w (f/f_0)} \tag{10}$$

where u^0 is the limiting mobility (in cm^2 V^{-1} sec^{-1}), r_w the Van der Waals radius (in Å) and f/f_0 the frictional ratio. Assuming that $f/f_0 = 1$ and calculating r_w by

$$r_w^3 = \frac{3 \cdot 10^{24}}{4\pi N_{Av}} \cdot \frac{\bar{M}}{\varrho} \tag{11}$$

where N_{Av} is Avogadro's number and ϱ the density (g cm^{-3}) of Ampholines*, we calculate $\bar{M} = 710$. An analogous calculation for Pharmalytes (ϱ was found to be 1.23 g cm^{-3}) gives $\bar{M} = 790$, which agrees well with the value mentioned above (763).

CONCLUSIONS

(1) In preparative isoelectric focusing the use of Pharmalytes as carrier

* We determined the density of Ampholines (pH 3.5–10) at 25° by the displacement method[28], using solid material obtained by drying a 40% Ampholine solution *in vacuo* over phosphorus pentoxide, with acetone as an inert immersion fluid. We found $\varrho_{25°} = 1.16$ g cm^{-3}.

ampholytes is to be preferred to that of Ampholines or Servalytes at $5.5 < pH < 8.5$. Outside this pH range the Ampholines are preferable.

(2) In analytical isoelectric focusing with narrow pH gradients the use of Pharmalytes or Servalytes is to be preferred to that of Ampholines at $pH < 6$, while Pharmalytes or Ampholines should be used instead of Servalytes at $pH > 6$. With broad pH gradients Pharmalytes are recommended.

(3) A combination of specific conductivity and buffer capacity data for focused fractions indicates that the molecular weight of the three carrier ampholyte systems generally decreases with increasing isoelectric point. The mean molecular weight increases in the order Servalytes, Ampholines, Pharmalytes.

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