gave substantially different release rates *in vitro*, yet both gave practically identical blood levels when tested in humans.

The fact that little or no drug was released in the first 3 hr. after drug administration, suggests that plain SETD can be added to the initial dose of the SETD–Glycowax S-932 combination without complications as far as product design is concerned.

Since less drug was released from the SETD–Glycowax S-932 (1:2) combination, a lower proportion of the wax appears necessary to obtain a medication that would meet the requirements for a more desired prolonged-release product.

The average physiologic availability of the drug from SETD–Glycowax S-932 combination tested *in vivo* was 59% after 72 hr. This low physiologic availability would reflect the incomplete release of the drug as indicated by urinary excretion data.

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Automated Differential Amperometric Analyzer

Application to Penicillin Determination

By JOSEPH BOMSTEIN, J. M. SHEPP, S. T. DAWSON, and W. J. BLAEDEL*

An automated, differential amperometric analyzer has been constructed, based on the tubular platinum electrode and a differential signal detection system. Its chief advantages are sensitivity to species which are oxidizable or reducible at the platinum electrode, and a linear relationship between concentration and signal. As applied to the iodimetric determination of several penicillins, precision is 0.7–2.6 per cent and accuracy ranges from 1.0 to −3.0 per cent.

CONSIDERABLE progress has been made in recent years in automating analytical laboratory processes. A number of reports deal with automation of 1 or more steps of analytical procedures, and outstanding success has been achieved industrially with the AutoAnalyzer (Technicon Instruments Corp., Chauncey, N. Y.), generally applying spectrometric techniques. A greater range of applications might be handled by combining the instrument's continuous-flow and chemical-physical processing capabilities with an electrometric detector. Redox analyses could then be performed. Such a system has been designed, constructed, and evaluated for the iodimetric determination of penicillins.

The method involves degradation of the penicillin with alkali or penicillinase, followed by iodimetric determination of the resulting penicilloic acid (1). A published procedure, in which a penicillin stream after hydrolysis and dialysis reacts with iodine to cause an absorbance decrease (2), has been modified and used to prove the practicality of an amperometric technique.

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EXPERIMENTAL

Differential Electrode Detection System.—The detector is a differential electrode system based on the tubular platinum electrode (TPE) (3, 4). Figure 1 shows the pair of platinum electrodes and the saturated calomel reference electrode. The sample stream enters one side of the Y-tube, flowing past a pulse suppression overflow tube, then through 0.5 in. length of platinum tubing (i.d. 0.06 in., o.d. 0.07 in.) which serves as 1 arm of a Wheatstone bridge. (See TPE_1 in Fig. 2.) The reference stream, which serves the purpose of blank compensation, flows past a second overflow tube, then through a second TPE identical to the first. This is the arm of the bridge TPE_2, in Fig. 2. The two streams combine to give a conducting path for current flow through the saturated-calomel reference electrode (SCE), physically separated from the flowing stream by a porous glass wall (code 7730 Vycor, Corning). Impurities introduced by diffusion into the SCE are displaced by a flow of saturated KCl/Hg_2Cl_2. The ground connections are added to improve electrical stability.

Bridge Circuit.—Figure 2 shows the schematic diagram of the bridge to which the signal from the electrodes is applied. Except for the replacement of the microvoltammeter and recorder by the VOM-6 recorder (Bausch & Lomb, Rochester, N. Y.) alone, it is identical to the bridge discussed by Blaedel and Olson (3). Initial balance is obtained

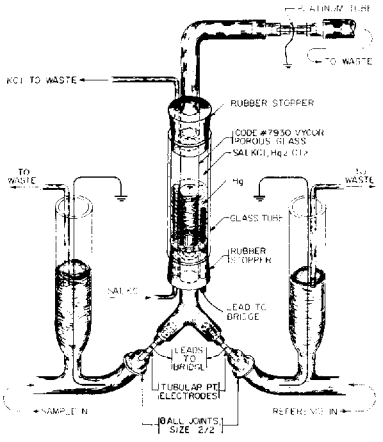

Fig. 1.—Electrode system.

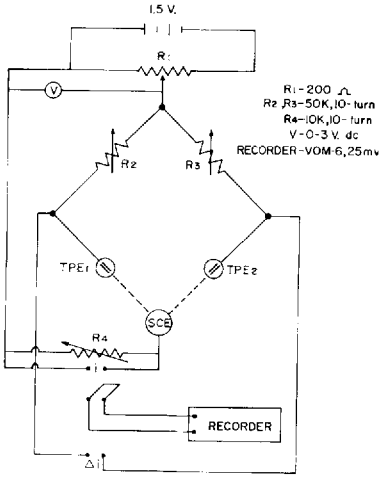

Fig. 2.—Bridge circuit. Key: —, wiring; – – –, solution (conducting) path.

by adjusting R_2 and R_3 while the blank solution is flowing through both TPE's. R_1 is set to a potential suitable for the amperometric measurement of iodine, as determined by the nature of the chemical system. When the sample replaces the blank in TPE_1, the balance between the 2 diffusion currents is disturbed, resulting in a displacement of the recorder pen when the switch is closed in the Δi-position. Closing the switch in the i position provides a convenient way to measure total current flow in order to select the proper recorder range. Impedance matching with the VOM-6 recorder requires the use of a millivolt range.

Pulse Suppression.—The reagent pump in this automated analyzer is peristaltic, and capable of long-term operation with excellent reproducibility over a wide range of speeds. The peristalsis, however, causes a pulsing flow of the reactive species through the electrodes, inducing a corresponding pulsing electrical signal at the recorder. Even careful matching of blank and sample channels is not adequate to remove the pulse effect when high recorder sensitivity is used. In order to provide a smooth base-line and sample record, a pulse suppression system is introduced. A flow-control pump (model PA-56, New Brunswick Scientific Co., New Brunswick, N. J.) is installed at the exit end of the system, pumping out at a rate less than the combined rate of the reagent, blank, and sample influent streams. The resulting difference in pressure causes an overflow from each channel into a chamber (10-ml. syringe body) which is open to the atmosphere at the other end. The liquid head is held at a predetermined level by pumping off the excess liquid to waste through the same pump used to supply electrolyte to the reference electrode. (See Figs. 1, 3, 4.)

Application to Penicillins.—The flow system for determining penicillins is shown in Fig. 3, and the relationship among the basic units is shown in Fig. 4. For test purposes, samples were fed manually, omitting any programming device. The reagent pump is the Harvard model 600-1200 (Harvard Apparatus Co., Dover, Mass.) having dual peristaltic channels pumping 180° out of phase. To accommodate the 8 (standard instrument) pump tubes required, an adjustable slotted rack is mounted on top and is positioned to hold 4 tubes tautly in each channel. (See Fig. 5.) Penicillin solution is continuously aspirated in a single tube and is split at a tee before passing through sample and blank lines in the Harvard pump. Successive samples are separated by allowing air to enter the pick-up tubing. In order to insure equal pump rates in blank and sample streams, the reagents are paired in each channel, *e.g.*, both KIO_3 streams are pumped in the same channel, etc. Despite this precaution, a small time difference may arise in the arrival of the streams at the TPE's. This difference

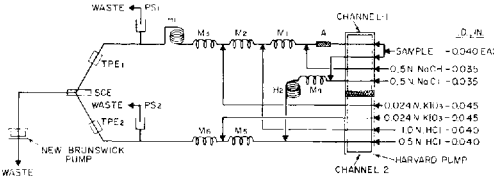

Fig. 3.—Iodimetric determination of penicillins.

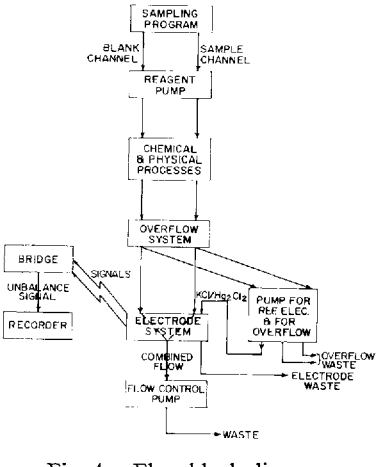

Fig. 4.—Flow block diagram.

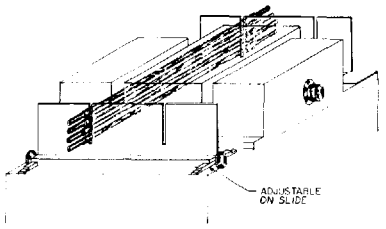

Fig. 5.—Pump modification.

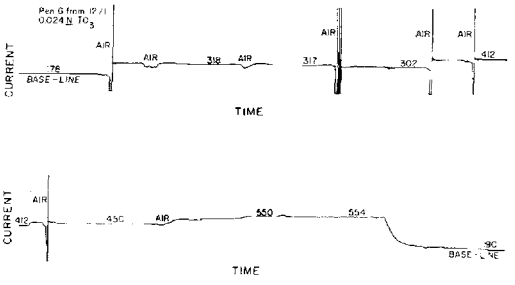

Fig. 6.—Typical amperometric recording.

can be minimized by extending or shortening 1 path as required, by means of a glass-in-Teflon telescoping sleeve arrangement at A (Fig. 3).

On the sample side, NaOH is added, and 3 large mixing coils at M_1 permit hydrolysis to proceed for 15 min. HCl is added to neutralize the alkali and to provide enough of an excess to liberate all available iodine from the KIO_3 which is added next. M_2 and M_3 are small mixing coils. The stream passes through a 12-min. holding coil H_1 to permit iodination, and is finally pumped through TPE_1, and SCE to waste. On the blank side, the sample is first mixed (mixer M_4, holding coil H_2) with NaCl equivalent to that produced on the sample side by the reaction of alkali with acid. Excess acid is added next, mixed at M_5, and KIO_3 is added finally to produce the same concentration of iodine at TPE_2 that would exist at TPE_1 in the absence of hydrolyzed penicillin. M_4, M_5, and M_6 are single mixing coils, and H_2 is made long enough to equalize the total path length of both streams. All mixers and holding coils are standard instrument parts. Tubing up to M_3 on the sample side and M_6 on the blank side is standard instrument transmission tubing; beyond these points the system is built of glass and Teflon to minimize absorption of iodine, except for the portions carrying the streams to waste.

The TPE potential is set at −50 mv. with respect to the SCE, recorder range at 25 mv. full-scale, and resistors R_2, R_3 at about mid-range. Reagents are those indicated in Fig. 3, except that the iodate contains 13.3 Gm. of KI per liter, required for the reaction $IO_3^- + 5\ I^- + 6\ H^+ = 3\ I_2 + 3H_2O$. Standards are prepared to cover the range 200–500 mcg. penicillin/ml. (Other operating conditions could have been chosen to accommodate lower or higher concentrations.) Adjustment of the pumps is important, and is accomplished as follows. The Harvard pump is set for 24 r.p.m., and the platen is moved toward the pumping fingers until flow through the tubing is just perceptible. The platen control knob is tightened 2 divisions more and is then locked in place. The New Brunswick pump is operated at 56 r.p.m., and the backing plate is tightened until the liquid levels in the pulse-suppressors are barely rising. The plate is then locked in position. The sampling pattern for penicillin determinations was chosen to provide maximum stability at the electrode and to minimize mixing between successive samples. Data reported here were obtained with the following manual program: sample aspiration, 2 min.; air, 15 sec.; sample, 5 min.; air, 15 sec. These 4 steps are repeated for each succeeding sample.

RESULTS

Penicillin G, oxacillin, phenethicillin, and ampicillin were determined successfully by the amperometric technique. Data were obtained by measuring the differences between diffusion current differentials recorded for samples and base-line, the latter recorded when samples were replaced by water in both channels. A typical trace is shown in Fig. 6.

Table I shows good precision and accuracy for several determinations on penicillin G, oxacillin, phenethicillin, and ampicillin, compared to Auto-Analyzer values determined colorimetrically (2). Instrumental response is linear (Fig. 7), plots of diffusion current against sample concentration giving straight lines within experimental error. Under these conditions, sensitivity is about 10–15 mcg./ml., and a range of 1–2 mcg./ml. appears attainable.

Data for the other penicillins show respective precision and accuracy of 1.0 and −0.2% for oxacillin, 0.7 and 1.0% for phenethicillin, and 2.6 and −3.0% for ampicillin. Methicillin could not be analyzed, probably because of its instability in acid solutions (5).

DISCUSSION

Advantages and Disadvantages.—Two major advantages accrue to the amperometric system: (*a*) linearity, the signal arises from the diffusion current, which is linear with concentration, and this eliminates the spectrometric problem of working with a logarithmic function; (*b*) measurement of spectroscopically inactive species which are reducible or oxidizable at the platinum electrode. Specificity may be obtained by proper choice of applied potential, electrode material, masking reactions, and solvent systems.

The measurement of a difference signal also permits simultaneous handling of the sample and its blank. In principle, the same advantage may be utilized in spectrophotometric analysis, although in practice this technique has not yet been widely applied in routine determinations.

Several difficulties have also been encountered with the amperometric system. Some are due to the choice of iodimetry for the application, but some are inherent in the electromechanical system. Chief among the latter are: (*a*) peristaltic pulsing. As discussed earlier, suppression of pulsing results in some experimental complexity. Considerable improvement in sensitivity was available for the

TABLE I.—PRECISION AND ACCURACY

Precision			
Penicillin G, u./mg.	Oxacillin, mcg./mg.	Phenethicillin, mcg./mg.	Ampicillin, mcg./mg.
1345, 1400	894, 894	897, 920	826, 826
1319, 1325	833, 826	879, 878	820, 852
1337, 1343	874, 872	883, 878	853, 803
1375, 1375	914, 914	883, 878	776, 758
1391, 1388	871, 871	862, 861	824, 815
1315, 1361	878, 874	847, 858	860, 831
1296, 1299	852, 813	870, 865	908, 888
1322, 1365	827, 848	883, 884	841, 903
1380, 1293	852, 850	811, 816	880, 875
1368, 1313	871, 854	820, 830	...
1351, 1283	852, 843	805, 815	...
Rel. S. D. 2.3%	1.0%	0.7%	2.6%

Accuracy[a]							
Penicillin G, u./mg.		Oxacillin, mcg./mg.		Phenethicillin, mcg./mg.		Ampicillin, mcg./mg.	
Found	AutoAnal.	Found	AutoAnal.	Found	AutoAnal.	Found	AutoAnal.
1373	1385	894	835	909	885	816	860
1322	1380	828	835	879	860	844	875
1340	1410	872	865	883	885	813	845
1375	1445	912	875	880	885	761	835
1390	1430	873	890	861	850	825	885
1338	1450	878	875	853	850	851	835
1298	1320	833	855	868	860	904	885
1344	1340	838	855	883	870	881	895
1337	1335	851	850	...	...	882	895
1341	1335	863	885	...	...	...	...
1317	1345	846	890	...	...	...	...
Mean error −37		−2		+9		−26	
Rel. error −2.7%		−0.2%		+1.0%		−3.0%	

[a] Averages of duplicates.

penicillins, but it is likely that the ratio of pulse-height/signal imposes a limitation. (*b*) Sample diffusion. In extended systems, such as is required in the determination of penicillins, diffusion occurs between adjacent samples unless some form of mechanical separation is introduced. When no separation is used, a steady state cannot be achieved, even with 10 to 15-min. sampling times. When air is introduced, a steady state is reached in about 4 min. This period, added to the 2-min. scrub, limits the throughput for the penicillin determinations to not more than about 8 samples/hr. This compares with the effective rate of 20/hr. for the colorimetric method (2). (*c*) Timing. Careful control is required to insure that sample and appropriate blank reach the electrodes simultaneously. The extent to which they are out-of-phase has a strong influence on the time required to reach a plateau in the diffusion current.

Effects of Iodate Concentration.—Although 0.004 *N* iodate is sufficient to react stoichiometrically with the highest penicillin concentration used (500

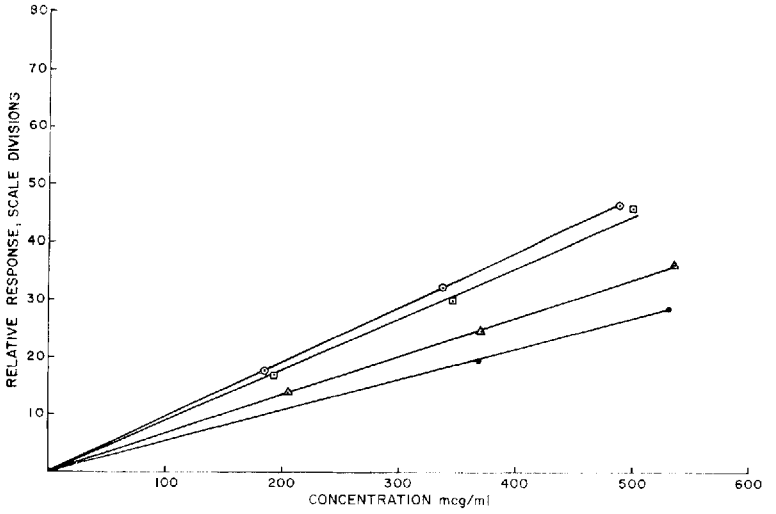

Fig. 7.—Instrument response for penicillins. Key: ⊙, phenethicillin; ⊡, ampicillin; ⧋, penicillin G; ●, oxacillin.

mcg./ml.), a deviation from linearity between response and concentration is observed at this concentration. All published iodimetric methods for penicillin require a greater-than-stoichiometric iodine-to-penicillin ratio; the reason has not been reported in the literature.

For the 4 penicillins studied, best linearity is obtained with 0.016 *N* iodate for penicillin G and oxacillin, and 0.024 *N* iodate for phenethicillin and ampicillin. At much higher iodate concentrations, deviations from linearity become apparent, possibly due to the increased influence of substitution reactions.

Iodine absorption on the tubing walls is minimized by using an all-glass-Teflon system beyond the point of acidification of iodate. Nevertheless, without air-scrubbing, visible deposits of iodine accumulate on the glass. Deposits also build up in the porous glass wall isolating the calomel electrode, but these can be washed out at the end of each working day. When more extensive washing appears desirable, 3 *N* HNO_3 is pumped through the entire system for 3 hr.

The influence of iodide ion on the penicillin method is minimized by holding a high concentration of excess iodide in all streams. In view of the current belief that the platinum electrode has an adsorbed I^- layer (6), the iodide is considered important to avoid electrical drift.

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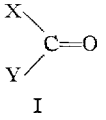
Notes

Molecular Orbital Localization Energies and Carbonyl Nucleophilic Reactivity

By LEMONT B. KIER

Molecular orbital anion localization energies have been calculated for a number of carbonyl group-containing compounds using the LCAO–MO Hückel procedure and the ω-technique. These energies have been found to reflect very adequately the summation of electronic effects from neighboring atoms to the carbonyl group as evidenced by the excellent correlation of the energies calculated with the rate constants of base catalyzed hydrolysis.

THE RELATIVE rate of nucleophilic reactivity of a carbonyl group-containing system such as I can be of great importance from the standpoint of



I

drug activity and drug stability, as well as contributing to structural and mechanistic theory. Classical resonance theory has dictated that a balance of mesomeric and inductive effects derived from atoms X and Y in I should facilitate or retard the ease of hydroxyl ion attack on the carbonyl carbon, this becoming the rate-determining step in the reaction sequences. The contributions of X and Y manifest themselves in the form of an energy barrier referred to as the free energy of activation, $\Delta F^{\ddagger}$. It has been assumed that, as a good approximation, $\Delta F^{\ddagger}$ is composed chiefly of $\Delta E^{\ddagger}_{\pi}$, the change in π electronic energy between the unreacted molecule and the transition state (1). In comparing the relative rates of reaction in a closely related set then the relationship holds

$$-RT \log \frac{k_2}{k_1} = [(\Delta E^{\ddagger}_{\pi})_1 - (\Delta E^{\ddagger}_{\pi})_2] \quad \text{(Eq. 1)}$$

The evaluation of $\Delta E^{\ddagger}_{\pi}$ demands a consideration of the nature of the transition state, or a reasonable model of it, in order to base the calculations on physical reality. Among several models proposed for the transition state, the σ complex or the Wheland intermediate has appeared to be the most satisfactory simple approach in the quantum mechanical study of chemical reactivity (2). In the case of the carbonyl carbon subjected to nucleophilic attack, the Wheland intermediate is a carbon surrounded by 4 bonds, each containing 2 electrons, III.

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