Titration Assembly and Experimental Procedure for Accurate pH Stat Measurements of Substrate Hydrolysis Rates in Blood Plasma

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A study was undertaken to establish an experimental procedure whereby accurate rates of enzyme-catalyzed substrate hydrolysis could be measured with a pH stat when solutions of blood plasma are used as the enzyme source. pH stat procedures which neglect the effects of plasma deterioration and loss of CO_2 from plasma solutions yield apparent hydrolysis rates which are faster and slower, respectively, than the true rates. To overcome the problems of anomalous substrate hydrolysis rates and apparent nonstoichiometric titrations, the plasma solutions were prepared aseptically and the titration assembly from a Radiometer pH stat was modified to effect a virtually closed system. Hydrolysis rate data are presented for acetylcholine, benzoylcholine, and phenyl butyrate in 0.5 percent solutions of human blood plasma to demonstrate the utility of the proposed experimental procedure and the modified pH stat titration assembly.

Solutions of whole human blood plasma are useful and convenient media for studying the *in vitro* enzymic cleavage of a number of drugs (1–7). However, they are seldom used in kinetic studies which utilize the pH stat for the determination of hydrolysis rates. This is apparently due to the labile nature of blood plasma (8). When exposed to air, solutions of blood plasma are prone to deterioration and loss of CO_2. Plasma deterioration results in a spontaneous increase in acidity, whereas loss of CO_2 decreases acidity. Thus, either effect may cause sizable errors in studies where hydrolytic processes occurring in blood plasma solutions are followed potentiometrically. This report describes a pH stat titration assembly and an experimental procedure useful for circumventing these errors.

Most studies which utilize blood plasma solutions as the enzyme media employ spectrophotometric (3, 4, 6, 7, 9, 10) or manometric (11–13) means of following the kinetics of substrate hydrolysis. Although capable of considerable precision, both methods have limitations. The spectrophotometric method is applicable only (*a*) to substrates with convenient absorption spectra which are distinguishable from those of their respective products; (*b*) to reaction mixtures devoid of extraneous substances which interfere with the assay of the substrates or products; and (*c*) to relatively low concentrations of substrate and enzyme. The manometric method, in addition to being laborious and time consuming, requires precise control of the CO_2 content of the reaction mixture. Both methods require the use of added buffers and, because of this, the enzymic reactions occur in an environment which differs from the physiological state of blood plasma.

Continuous potentiometric titration at constant pH without the use of additional buffers provides a convenient method for following enzymic hydrolysis reactions, especially of substrates with indistinguishable ultraviolet or visible absorption spectra. With the recent development of commercial automatic pH stats, this method has become increasingly useful (14–16, 18). Several factors which may influence pH stat results have been mentioned, but these are of little significance when the more recently developed techniques and apparatus are employed (17–21). The main objection to the method has concerned dilution of the reaction mixture by the titrant, but this effect can be overcome by keeping the volume of titrant small with respect to the volume of the reaction mixture or by correcting the kinetic data mathematically (22). When solutions containing blood plasma are used as the enzyme media, two additional effects must be considered. These are (*a*) the spontaneous production of an acid due to deterioration of the plasma, and (*b*) the loss of CO_2 from the plasma solution. Both effects, to our knowledge, are often either overlooked or inadequately considered in studies

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which utilize the pH stat to follow hydrolytic processes occurring in blood plasma solutions.

It has been observed in these laboratories that nonsterile solutions of blood plasma slowly decrease in pH on standing in a closed vessel where the CO_2 content of the solutions is kept constant. This effect is presumably due to bacterial deterioration of some ingredient of plasma, the result of which is a spontaneous production of an acid. Although the identification of the acid as well as its source have not been an objective of this study, the possible effect of the acid on pH stat results is of paramount importance. The pH stat plots the course of a hydrolysis reaction as a function of time by automatically titrating with a standard base solution the hydrogen ions produced as the reaction proceeds at a constant pH. Therefore, the acid produced as a result of plasma deterioration will obviously contribute to the rate as well as to the extent of the hydrolysis reaction. It was found that the acid may be produced in such large amounts that it could completely mask the hydrolytic process of substrates which hydrolyze at relatively slow rates. It is apparent, therefore, that, in order to obtain reliable data with the pH stat, adequate steps should be taken to minimize bacterial contamination during all phases of handling of the blood plasma.

The presence of large amounts of CO_2 in solutions of blood plasma presents another problem in pH stat determinations of hydrolysis rates. If the reaction is carried out in an open vessel, CO_2 will rapidly diffuse from the plasma solution to the atmosphere. The loss of CO_2 results in an increase in the basicity of the system which subsequently contributes to the titration of the hydrogen ions produced by the hydrolysis reaction. The conventional use of a nitrogen flush, while useful in systems completely free of CO_2 and bicarbonates (19), accelerates the loss of CO_2 by expelling it out of solution at a faster rate. Thus, to maintain constant CO_2 content of blood plasma solutions, the hydrolysis reactions should be carried out in a closed system.

In preliminary work with the pH stat using a 0.5% solution of blood plasma as the reaction medium, several attempts were made to completely purge the solution of CO_2. This was done by bubbling nitrogen gas through the plasma solution. In every instance, the pH of the solution first rose quite rapidly to about 9.5 where it remained constant. Since there was no further rise in pH, it was assumed that no more CO_2 could be removed from the solution. However, upon returning the pH of the solution to 7.4 and then continuing with the nitrogen flush, the pH once more rose to about 9.5, this time at a slower rate than previously. The same procedure was repeated several times with similar results, except that it took longer for the pH to reach 9.5 with each succeeding trial. While it may have been possible to remove all of the CO_2 from the solution, the procedure would be considerably difficult and time consuming. In addition, the rise in pH and change in plasma composition resulting from such treatment could affect the activity of the enzymes in blood plasma.

On the basis of the foregoing discussions, it seems obvious that adequate control over plasma deterioration and loss of CO_2 must be exercised to obtain accurate hydrolysis rate data with the pH stat. The manner in which this is achieved, that is, the use of an aseptic procedure for preparing the blood plasma solutions and the design of a virtually closed titration assembly, is described in this report.

EXPERIMENTAL

Materials—One-half percent solutions of whole human blood plasma[1] were prepared by an aseptic procedure using sterile, pyrogen-free, isotonic saline solution (0.15 *M* NaCl)[2] as the dilution medium. Alcoholic stock solutions of acetylcholine iodide,[3] benzoylcholine chloride,[3] and phenyl butyrate[3] in a concentration of 0.04 *M* were used as substrates. The standard base titrant was 0.02 *M* NaOH which was prepared free of CO_2 in the usual manner.

Apparatus—A Radiometer pH stat[4] was used. It consisted of a type TTT1 automatic titrator, SBR2c recorder, SBU1 syringe buret, and TTA3 titration assembly which was modified to effect a virtually closed system. The modified titration assembly is shown in Fig. 1.

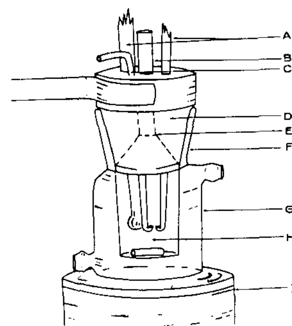

Fig. 1—Diagram of modified pH stat titration assembly described in the text.

The reaction vessel (H) is equipped with a water jacket (G) and a ground-glass joint (F) which pro-

[1] Philadelphia Serum Exchange, Philadelphia, Pa.
[2] Abbott Laboratories, North Chicago, Ill.
[3] Eastman Kodak Co., Rochester, N. Y.
[4] Radiometer, Copenhagen, Denmark.

vides an air-tight fit on the tapered Teflon plug (D). The Teflon plug holds the electrodes (A), the titrant delivery tube (C), and an inlet tube (B) 6 mm. in diameter and concentrically located on the plug. The bottom of the plug is tapered inward to form an inverted cone which peaks at the bottom of the inlet tube. The vessel is so made that when 20 ml. of plasma solution is used and the vessel is placed in position on the plug, the level of the solution just covers the bottom end of the inlet tube. A drop of light liquid petrolatum is then placed in the inlet tube to form a thin layer of oil on the surface of the plasma solution (E). By confining the oil layer only to the small opening of the inlet tube, the tendency is minimized for any oil-soluble component of the reaction mixture to partition into the oil. The inlet tube also serves as a means of introducing the enzyme, substrate, and other additives into the system by the use of a hypodermic syringe and needle. Stirring of the reaction mixture is done by means of magnetic stirrer (I) located beneath the vessel.

Preparation of the Blood Plasma Solutions—Blood plasma is usually supplied frozen in 120- to 150-ml. quantities. When defrosted and exposed to air, it deteriorates quite readily. Since plasma deterioration is apparently due to bacterial contamination, it is expected that antibacterial agents such as a streptomycin and sulfathiazole might be used to alleviate this condition (23). However, it was felt that the addition of such agents might further complicate the system, for example, by reducing the enzyme activity or by interfering with the potentiometric titration, and that the use of an aseptic procedure for preparing the plasma solutions might be more practical.

The procedure is as follows. The plasma is first defrosted, mixed well, and then transferred in a sterile room to serum flint vials in 1–2-ml. portions. The vials are sealed with rubber closures and placed in a freezer at $-18°$. Dividing the plasma into small portions eliminates the need to defrost and refreeze the bulk plasma every time a sample is removed and, thus, reduces the chances for the plasma to deteriorate. At the time of use, a vial of plasma is removed from the freezer and allowed to thaw at 37°. One milliliter of the plasma is transferred under sterile conditions to a 100-ml. volumetric flask and brought up to volume with sterile, pyrogen-free, isotonic saline solution to make a 0.5% solution of blood plasma. This solution is stable up to 24 hr. with respect to its enzyme activity and relative freedom from deterioration by bacteria.

Cleaning of the Titration Assembly—Immediately prior to the start of a run, the reaction vessel, as well as all parts of the titration assembly which normally come in contact with the plasma solution, are cleaned first by washing with a 70% solution of ethanol, and then thoroughly rinsing with the sterile saline solution. They are then wiped dry with a sterile cotton gauze.

Procedure for a Typical Kinetic Run—Twenty milliliters of the 0.5% blood plasma solution is transferred to the reaction vessel by means of a sterile pipet and the vessel is placed in position on the pH stat. A drop of light liquid petrolatum is placed on the surface of the solution through the inlet tube. The speed of the magnetic stirrer is adjusted so that stirring is just sufficient to cause rapid flow of the solution past the electrodes without creating cavitation and foaming. Constant-temperature water ($37 \pm 0.2°$) is circulated through the jacket of the reaction vessel.

The plasma solution is first adjusted to pH 7.4 with the pH stat. The pH stat is set so that, as the pH of the solution approaches 7.4, the titrant is added at a rate essentially the same as that required by the reaction. This procedure quickly titrates the free acids present, prevents the possibility of the automatic titration falling behind the reaction, and eliminates large "step-effects" due to the addition of excess titrant. The pH stat is left to run until no titrant is consumed for at least 15 min. with the pH remaining constant at 7.4. When this occurs, the system is said to have achieved pH equilibrium. The recording pen is returned to zero and the syringe buret is refilled with titrant.

The kinetic run commences when 0.1 ml. of stock substrate solution is added to the plasma solution. The pH stat is adjusted once more to compensate for any change in reaction rate, and the reaction is allowed to proceed either to completion or until sufficient data are obtained.

Several investigations were made using 0.5% solutions of blood plasma with and without substrates to determine the effects of (*a*) plasma deterioration, (*b*) the loss of CO_2 from the reaction mixture, and (*c*) a virtually sterile and closed reaction system in which the effects of both plasma deterioration and loss of CO_2 are minimized.

The magnitude of the effect of plasma deterioration was determined by the use of two solutions of blood plasma: one which was freshly prepared and the other which was left exposed to air at room temperature for 2 hr. No precautions were taken to maintain the sterility of these solutions. To eliminate the effects due to the loss of CO_2 from the reaction mixture, the reactions were carried out in the modified titration assembly.

The effect due to the loss of CO_2 from the reaction mixture was determined by the use of a sterile solution of blood plasma in the titration assembly supplied with the instrument. This assembly, not being a closed system, allows CO_2 to escape freely from the reaction vessel. A 0.02 *N* HCl titrant was used to follow the rate of base production. After a specified time, a nitrogen flush similar to the one described by Applewhite (20) was introduced to show the in-

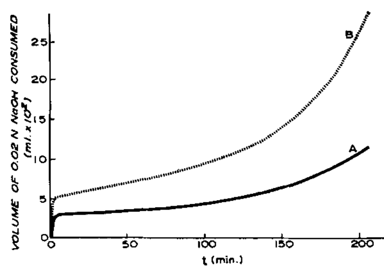

Fig. 2—Plots showing the effect of blood plasma deterioration. The increased rate of base consumed reflects the rate at which acid is spontaneously produced when plasma degrades. Curve A *was obtained using a freshly prepared plasma solution. For curve* B, *a plasma solution which had been aged for 2 hr. at room temperature was used. (Plasma concentration,* 0.5%.)

creased rate of base production due to a faster loss of CO_2.

Finally, the utility of the proposed experimental procedure and the modified pH stat titration assembly was demonstrated. Solutions of 0.5% blood plasma, prepared in the manner described, were used as the enzyme media with acetylcholine, benzoylcholine, and phenyl butyrate as substrates. To check the accuracy of the results obtained with the pH stat, parallel runs were made with the latter two substrates on a Cary model 15 spectrophotometer according to the method of Kalow *et al.* (9).

All experiments were conducted at pH 7.4 and 37°. For purposes of comparison, the hydrolysis rates for benzoylcholine and phenyl butyrate were determined from apparent zero-order rate plots of the initial stages of the hydrolytic processes.

RESULTS AND DISCUSSION

The effect of the acid produced as the result of plasma deterioration is shown by curves A and B in Fig. 2.

Curve A was obtained from the freshly prepared, but nonsterile solution of blood plasma. During the early stages of the run, the rate of acid production appears linear and the amount of acid produced is relatively insignificant. However, as the run progresses, the acid is produced at an increasing rate. The system behaves as if the acid exerts a catalytic effect which accelerates the production of more acid.

Curve B was obtained using a similar solution of blood plasma except that it was left to stand in an open vessel at room temperature for 2 hr. The higher initial uptake of the base titrant indicates that more free acids were present in this solution at the beginning of the run than in the fresh solution of plasma. The plasma had obviously undergone some deterioration during the 2-hr. period to produce the additional acid. Apparently, due to autocatalytic effects, the rate of acid production is noticeably faster at corresponding time periods than in the previous run with the fresh plasma solution.

During the initial stages, the rate of uptake of the base titrant is 2.0 μm./L./min. and 6.5 μm./L./min. for curves A and B, respectively. After 2 hr., the rates increase markedly to 9.6 μm./L./min. for curve A and 18.8 μm./L./min. for curve B. Thus, it is highly probable that the effect due to plasma deterioration might largely influence or even mask hydrolytic processes especially of substrates which hydrolyze slowly.

Figure 3 shows the effect due to the loss of CO_2 from the reaction mixture. The rate of CO_2 loss is 2.8 μm./L./min. and it is linear in the range studied. It is slightly greater than the initial rate of plasma deterioration for the freshly prepared plasma solution, but less than that for the plasma solution which was aged for 2 hr. At the point N_2 on the curve, a nitrogen flush was introduced into the system. This resulted in a more rapid loss of CO_2 as indicated by the higher rate of uptake of the acid titrant.

The consequences of plasma deterioration and loss of CO_2 are opposite in effect. Usually, the rate of CO_2 loss exceeds the rate of plasma deterioration during the early stages of a run. However, as the run progresses, the rate of plasma deterioration accelerates markedly, presumably by autocatalysis, and becomes far greater than the rate of CO_2 loss. To demonstrate these effects, which are shown in Fig. 4, two separate pH stat units were used with a common titration assembly. One unit contained a 0.02 *N* NaOH titrant which followed the rate of plasma deterioration while the other unit, with a 0.02 *N* HCl titrant, followed the rate of CO_2 loss.

The data represent the difference between the amounts of base and acid titrants consumed during the run. It can be seen that up to about 2 hr., the rate of CO_2 loss predominates. Beyond this time, the rate of plasma deterioration becomes increasingly faster and gradually exceeds the rate of CO_2 loss. Thus, the effect on the over-all hydrolysis rate of a substrate which cleaves slowly would probably be

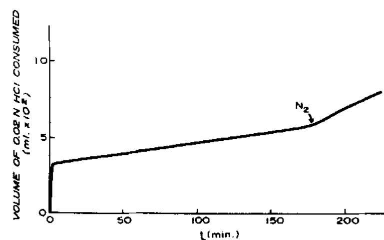

Fig. 3—Plot showing the effect of CO_2 loss. The rate of base production appears linear in the time range studied. At the point N_2 *a nitrogen flush was introduced into the system. The increased rate of base production is due to the faster rate at which CO_2 is expelled from solution. (Plasma concentration, 0.5%.)*

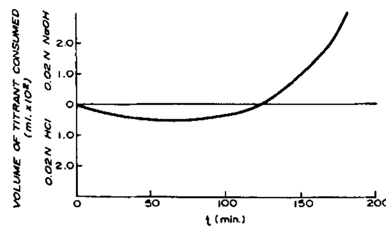

Fig. 4—Data showing the simultaneous effects of plasma deterioration and CO_2 loss with time. Up to about 2 hr., the effect of CO_2 loss predominates, after which time the effect of plasma deterioration becomes more pronounced. (Plasma concentration, 0.5%.)

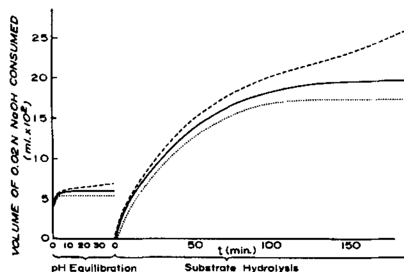

Fig. 5—Plots showing the effects of plasma deterioration and CO_2 loss on the enzymic hydrolysis rate of acetylcholine. Curve A *shows the effect of plasma deterioration, curve* C *the effect of CO_2 loss, and curve* B *the true hydrolysis rate of the substrate. Curve* B *was obtained by use of the experimental procedure described in the text. (Plasma concentration, 0.5%.)*

TABLE I—INITIAL HYDROLYSIS RATES OF ACETYLCHOLINE, BENZOYLCHOLINE, AND PHENYL BUTYRATE IN 0.5% HUMAN BLOOD PLASMA DETERMINED AT DIFFERENT EXPERIMENTAL CONDITIONS

Substrate →	Hydrolysis Rate, μm./L./min. — Acetylcholine			Benzoylcholine			Phenyl Butyrate		
Method → / Experimental Condition	pH Stat	Spectrophotometer	% Dev.	pH Stat	Spectrophotometer	% Dev.	pH Stat	Spectrophotometer	% Dev.
Closed titration assembly with nonsterile plasma solution. (Effect of plasma deterioration.)	57.3	...	+ 7.5	121	...	+2.5	106	...	+2.9
Closed titration assembly with sterile plasma solution.	53.1	...	...	118	120	...	103	104	...
Open titration assembly with sterile plasma solution. (Effect of CO_2 loss.)	47.6	...	−11.3	108	...	−8.5	93.1	...	−9.7

first a slowing down of the rate up to the 2-hr. period, followed by an acceleration of the rate. The hydrolysis reaction then appears to proceed beyond the stoichiometric level because the acid from plasma deterioration continues to be titrated.

Jensen-Holm (16), who was perhaps the first to note that a nonspecific acid production takes place in most tissue homogenates and blood solutions, reported that: "By keeping the system anaerobic (bubbling nitrogen through it for 20 minutes and then maintaining an atmosphere of nitrogen in a half-closed system) the spontaneous, unspecific acid liberation can be not only greatly reduced, but also often converted into a fairly constant base production." It seems apparent that by bubbling nitrogen through the system, he was driving off CO_2 (resulting in an increased basicity of the system) at a rate which was nearly the same or which exceeded the rate of acid production.

Figure 5 shows the effects of plasma deterioration and CO_2 loss on the hydrolysis rate of acetylcholine in a 0.5% solution of blood plasma. The figure also shows data which attest to the utility of the proposed experimental procedure and the modified pH stat titration assembly for minimizing these effects.

The effect due to plasma deterioration is shown by curve A. It can be seen that a pH equilibration could not be attained due to the slow but continuous titration of the acid produced. Upon the addition of acetylcholine, the hydrolysis reaction proceeds at a rate faster than normal because the acid resulting from the deterioration of plasma is titrated in addition to the acid produced by the hydrolytic process. Curve A also shows that the consumption of base titrant continues far beyond the stoichiometric amount equivalent to complete hydrolysis of the substrate. The excessive uptake of titrant is obviously due to the acid produced as the result of plasma deterioration.

Curve C shows the effect due to the loss of CO_2 from the reaction mixture. Although a pH equilibration seemed to have been attained prior to the addition of acetylcholine, the pH of the plasma solution was actually above 7.4 due to the loss of some CO_2 from the plasma solution. Since the pH stat does not begin to record the amount of titrant delivered until the pH of the solution is returned to 7.4, there is a slight lag between the time the substrate is added and the start of the titration. The hydrolysis reaction then proceeds at a rate slower than normal, and reaches a level below that for complete hydrolysis of the substrate. Obviously, the base formed as the result of CO_2 loss also serves to titrate the acid produced by the hydrolysis reaction. Thus, less of the base titrant is needed for the over-all reaction.

The data obtained using the proposed experimental procedure and the modified pH stat titration assembly are shown by curve B. The pH equilibration of the plasma solution is attained readily and remains stable until the substrate is added. The pH stat, which is now unaffected by errors due to plasma deterioration and loss of CO_2, plots the true course of the hydrolytic process and reaches an equilibrium when the amount of base titrant consumed is nearly the same as that theoretically required by the hydrolysis reaction.

The enzymic hydrolysis rates for benzoylcholine and phenyl butyrate, determined both by the potentiometric titration method (pH stat) and the spectrophotometric method (Cary model 15), are shown in Table I. The pH stat data for acetylcholine are also presented for the purpose of comparison. The "initial" rates were determined from the slopes of the essentially linear portions of the titrator plots in the initial 10-min. periods.

The results obtained by the use of the closed pH stat titration vessel and sterile plasma solutions correlate well with the spectrophotometric results. The results, which were influenced by the effects of plasma deterioration and the loss of CO_2, were higher and lower, respectively, than the results obtained spectrophotometrically. It will be noted that the percent deviation from normal becomes smaller the faster the hydrolysis reaction. For example, in the case of benzoylcholine which hydrolyzes 1.14 and 2.22 times faster than phenyl butyrate and acetylcholine, respectively, the deviation due to plasma deterioration is 2.5% and that due to the loss of CO_2 is 8.5%. On the other hand, in the case of acetylcholine, the slowest hydrolyzing substrate of the three, the deviation is 7.5% for plasma deterioration and 11.3% for CO_2 loss. Thus, while the effects of plasma deterioration and CO_2 loss may not be highly significant in studies of rapidly hydrolyzing substrates, they may have considerable influences on the hydrolysis of substrates which cleave at relatively slow rates.

SUMMARY

The effects of plasma deterioration and CO_2 loss may be significant in studies which utilize the pH stat for the determination of enzymic hydrolysis rates occurring in solutions of blood plasma. To minimize these effects, the plasma must be kept virtually free of bacterial contamination and the

reactions should be carried out in a closed system. A method for the aseptic preparation of blood plasma solutions and the utility of a closed pH stat titration assembly are described. Data are presented to show the utility of the experimental procedure for following the hydrolysis of substrates such as acetylcholine, benzoylcholine, and phenyl butyrate in solutions of blood plasma.

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Notes

Radiation and the Detoxication of Chemicals

By THOMAS J. HALEY* and L. KOSTE

The detoxication of benzoate and resorcinol has been investigated in irradiated animals. In both instances detoxication was inhibited from the third to the seventh post-irradiation days. The common denominator for this action of X-irradiation appears to be co-enzyme A and/or its substrates.

Exposure to X-irradiation is known to increase protein catabolism (1) and increase the free and dynamic glycine pools in the animal (2–4) but Schreier *et al.* (5) found no change in hippurate excretion during the first 3 days post-irradiation. However, Gerber and Remy-Defraigne (6) found that the irradiated liver synthesized more hippurate when exogenous glycine was excluded from the perfusion fluid than when it was present. Chiriboga (7) reported that whole body irradiation caused a decreased glucuronide excretion and suggested that further investigation was necessary to elucidate its role in the radiation syndrome. Inasmuch as both hippurate and glucuronide synthesis involve co-enzyme A and through it the detoxication of benzoate and phenolic compounds, we have investigated the detoxication of benzoate and resorcinol in irradiated rats.

METHODS AND MATERIALS

Hippurate excretion was determined in 18 Charles River CD strain female rats weighing 250 to 300 Gm. and glucuronide excretion was obtained with 6 similar rats. Total 24-hr. urine volumes based upon 137 collections was 22.1 ± 10.5 ml. with a range of 6.5 to 55.8 ml. Urinary output depended upon water consumption which also had the same degree of variation. Normal excretion values for both hippurate and glucuronide were obtained in nine separate experiments and control values after administration of either 100 mg./Kg. of sodium benzoate or 50 mg./Kg. of resorcinol intraperitoneally in three separate experiments after which the animals received 600 rads acute whole body X-irradiation. In all cases urine was collected over a 7-day period by the method of Haley *et al.* (8). Urinary hippurate was determined with an Aminco-Bowman spectrophotofluorometer using the method of Ellman *et al.* (9) and glucuronide was determined by the method of Mead *et al.* (10). Quadruplicated samples of each urine specimen were analyzed. The results were analyzed statistically by the Student *t* test for significance. The radiation characteristics of the industrial unit were: 250 KVP; 15 ma.; FOD 55 cm.; filters 0.21 mm. Cu inherent; 0.5 mm. Cu parabolic and 1 mm. Al; HVL 1.95 mm. Cu; dose rate measured in air, 24.97 rads/min. The machine was calibrated prior to and after each experiment with a Victoreen thimble *r*-meter and during exposure with a Radacon unit.

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