PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 282-288 (1978)

Distribution Kinetics of Urea Between Plasma and Red Blood Cells of Normal Human Blood[1] (40038)

M. JEEVANANDAM,[2] C. L. LONG,[3] AND J. M. KINNEY

Department of Surgery, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, New York 10032

Urea passes through all the membranes of mammalian organs without any apparent resistance and the $t_{\frac{1}{2}}$ for the entry of urea into the human red blood cells (RBC) from an isosmolar solution is 0.053 sec at 21° (1). In spite of this rapid transfer of urea a substantial variation between plasma urea and cell urea concentrations under physiological conditions had been reported (2-5). This concentration difference was postulated as due to the existence of urea in various states. However no attempts were made to distinguish between the freely exchangeable urea and any relatively bound urea. Model dialysis experiments (6, 7) suggest that the clearance rate of urea from plasma may not be the same as that from RBC. Using isotopic techniques we have studied the *in vitro* kinetics of urea distribution between these two blood compartments and the *in vivo* fractional turnover rates of labeled urea. There is no concentration difference in the exchangeable (free) urea content between the cell water and plasma water. The difference found between cell urea and plasma urea sp act in all our [^{15}N] experiments suggest the existence of more bound urea in cells than in plasma.

Materials and methods. (a) *In vivo study.* Healthy normal volunteers, with their written consent, were admitted to the Surgical Metabolism Unit of Columbia-Presbyterian Medical Center and placed on a metabolic balance program for two days. They were provided with regular diet containing enough nitrogen to maintain balance. On the third day, 100 mg of pyrogen free urea (98 atom % [^{15}N]) in 15 ml normal saline was injected intravenously. Just prior to the injection a control sample of venous blood was taken. After dose injection blood samples (10-15 ml) were withdrawn in heparinized syringes at 10, 30, 60, 90, 120, 180, 210, 300, 360, 420, 480, 540, and 660 min and immediately centrifuged to separate the plasma from the cells. In one subject (MS), two days after this study of the distribution of the injected [^{15}N]urea between RBC and plasma (MS-1), when the level of the labeled urea attained almost the base value, [^{15}N]-L-alanine was injected and the sp act of the endogenously produced [^{15}N] urea in RBC and plasma were determined (MS-2).

The RBC and plasma fractions were diluted with deionized water and deproteinized by the Somogyi procedure using $Ba(OH)_2$ and $ZnSO_4$. After centrifugation, the supernatant fractions were shaken for 15 min with 2 ml of permutite to remove the ammonia of the blood and then treated in duplicate with urease in a Conway micro diffusion system. One milliliter of saturated K_2CO_3 was added to liberate ammonia which was then trapped on a filter paper strip (7.0 × 0.3 cm) moistened with a few drops of 1 *N* H_2SO_4. Sufficient time (about 8 hr) was allowed for the diffusion of ammonia. The paper strip was then removed and treated with sodium hypobromite in a micro Rittenberg gas tube. The liberated nitrogen gas was analyzed for its isotopic content in a 60° mass spectrometer which is capable of giving a precision of about five parts per 100,000. This procedure of trapping ammonia in a filter paper and using a micro Rittenberg tube minimizes any air contamination and provides maximum response for the peak heights of masses 28, 29 and 32. The replicate measurements

[1] This work was supported in part by the National Institute of General Medical Sciences Grant No. GM-14546-09 and U.S. Army Medical Research and Development Command, Contract No. DA-49-193-MD-2552.

[2] To whom reprint requests should be sent.

[3] Present address: Dr. C. L. Long, Department of Surgery, Medical College of Ohio, Toledo, Ohio 43614.

0037-9727/78/1572-0282$01.00/0

always agree within 0.5%. Reference nitrogen gas (0.365±0.002 [^{15}N]atom %) was analyzed at regular intervals to check the stability of the instrument. Atoms % excess were determined by subtracting values for atom % [^{15}N] in control samples taken prior to dose injection. In some samples of JL-1 and JL-2, the [^{15}N] atom % excess in the whole blood was also measured to check the isotope distribution by material balance.

(b) *In vitro study*. Twenty milliliter of freshly drawn heparinized normal venous blood was kept in a water bath at 37±0.2° and stirred slowly with a magnetic stirrer. About 1 to 2 mg of [^{14}C] urea (sp act 0.27 μCi/mg) was dissolved in 0.1 ml saline and carefully mixed with the blood. The osmolality change due to the addition of this small volume of tracer solution should be insignificant and any change in the hematocrit may be considered negligible. Using [^{131}I] albumin as a marker we have found that almost complete separation of the plasma and cells was possible by spinning capillary hematocrit tubes (75 mm × 1.5 mm i.d.) filled with blood in a micro centrifuge (International Micro-Capillary Centrifuge, Model MB). Samples were withdrawn at 1, 5, 15, 45, 60, 90, 120 and 180 min intervals in hematocrit tubes of uniform i.d., one end of which were then sealed and spun for three min. After cutting with a file and discarding the interface, known lengths of hematocrit tubes containing plasma and cell segments were collected separately. They were then crushed and deproteinized by mixing with 2 ml deionized water, 1 ml 0.3 *N* $Ba(OH)_2$ and 1 ml 0.3 *N* $ZnSO_4$ (Somogyi procedure). After centrifugation, the clear supernatant was filtered and a 0.5 ml duplicate of the filtrate was mixed with 10 ml Bray's solution and counted in a liquid scintillation counter (Model Mark II, Nuclear Chicago Corp., Des Plaines, Ill). All samples were counted for at least 10^4 counts. Internal standards were used for efficiency calculations. The recovery of [^{14}C] urea during the Somogyi precipitation of the cells or plasma and any dragging of [^{14}C] urea by the proteins was checked independently by deproteinizing the cells or plasma each containing equal amounts of [^{14}C] urea and then counting as described before. No differences were found between the control (583±10 dpm) and the plasma (579±15 dpm) or cell samples (590±17 dpm), confirming the same recovery from deproteinizing cells and plasma as well as the absence of any drag of [^{14}C] of urea by the proteins.

Similar *in vitro* experiments were done with [^{15}N] urea, and the sp act (^{15}N atom % excess) in the separated cells and plasma were determined as described before in the *in vivo* study.

We have also measured the efflux of the labeled urea from the cells into the surrounding medium of plasma. A 25 ml portion of the fresh blood (PUN = 11.0 mg%, Hct = 30.1) was centrifuged for 20 min and the plasma was separated from the cells. One portion of the plasma was used to reconstitute the blood and the rest was analyzed for its water content. Another 25 ml original blood was equilibrated at 37° for 3 hr with about 1 mg of [^{14}C] urea dissolved in 0.1 ml normal saline. The labeled cells were then separated by centrifugation and mixed with sufficient original nonradioactive plasma to reconstitute blood. The efflux of [^{14}C] urea from the cells of this reconstituted blood was then determined as described before for the influx experiments.

The water content of the cells and plasma in all the samples was measured in duplicate by drying the diluted samples under vacuum at 70° to constant weight. The specific gravities of blood and plasma were also measured. The volume percent of water in cells (range 69.5–71.3) and plasma (range 92.5–93.7) corresponds to a water distribution ratio of 1.25–1.30 between equal volumes of plasma and cells with a mean (±SEM) value of 1.288±0.007.

Results. The time course of the disappearance of the injected urea from plasma and cells for a typical subject (BW) is illustrated in Fig. 1. The sp act of the plasma urea was consistently higher than that of the cell urea. Both the plasma and cell urea curves followed a two exponential decay. The slopes of the plasma and cell curves on a semilog scale are not parallel to each other but tend to converge during the initial period of about 3 hr and then diverge for all

the subjects studied. These differences are significant ($P < 0.01$) when examined by paired t tests. By least squares method the logarithm of sp act was regressed with time and the calculated slopes which are the fractional turnover rates of urea from plasma or cells are summarized in Table I. The sp act ratio between plasma and cells (P/C) was always found to be greater than one. There was a rather slow change in the P/C ratio during the study (Table I) and its range was about ±4% of the mean. The whole blood urea sp act measured in some of the samples from JL-1 and JL-2 were found to be lower than that of corresponding plasmas but higher than that of cells and the relative sp act in blood, plasma and RBC was 1.00:1.06:0.92.

In the *in vitro* experiments the addition of a tracer will have negligible effect on the membrane hydraulic conductivity or on the solvent flow through the membrane (8). Under these experimental conditions, the predominating factor is the diffusional flow of urea. Figure 2 shows the distribution ratio (Mean±SEM) of the radioactive [^{14}C] between equal volumes of plasma and cells as a function of time in influx (n=4) and efflux (n=4) *in vitro* studies. The diffusion of urea into the cell or out of the cell

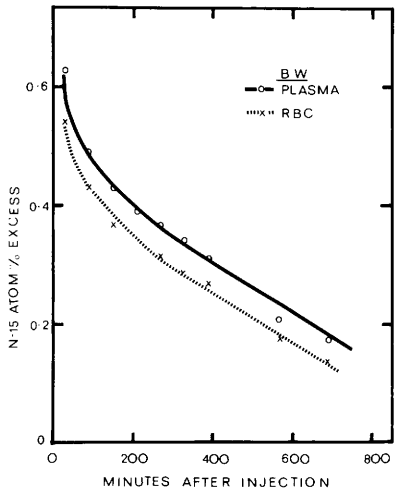

FIG. 1. Isotope concentration of plasma (○) and RBC (×) urea following intravenous injection of [^{15}N]urea in subject BW.

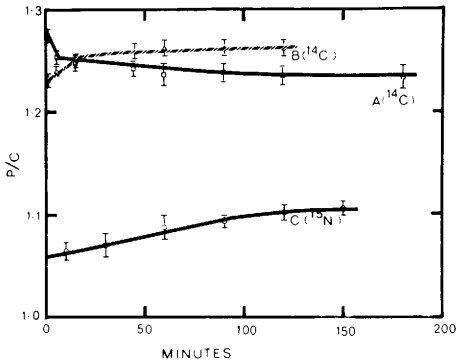

FIG. 2. The trend of change in the ratio of the tracer concentration (Mean±SEM) between Plasma (P) and RBC (C) with time (min) in *in vitro* studies: A: [^{14}C]urea, influx: n = 4. B: [^{14}C]urea, efflux: n = 2. C: [^{15}N]urea, influx: n = 4.

TABLE I. TURNOVER RATES OF UREA[a] FROM PLASMA (TRP) AND RED BLOOD CELLS (TRC) AND THE RANGE OF (P/C)[b] RATIOS.

		First (10–180 min)			Second (180–570 min)		
Subject	Sex	TRP	TRC	P/C Range	TRP	TRC	P/C Range
JL-1	M	37.1 (0.82)[c]	32.4 (0.78)	1.170–1.087			
JL-2	M	80.2 (0.93)	78.1 (0.93)	1.191–1.167			
GR	M	18.9 (0.96)	11.8 (0.93)	1.259–1.187			
MS-1	M	28.4 (0.93)	26.5 (0.91)	1.106–1.080	12.1 (0.97)	14.2 (0.92)	1.080–1.120
MS-2	M	64.2 (0.99)	50.4 (0.96)	1.176–1.073	13.9 (0.99)	14.1 (0.94)	1.073–1.110
KK	F	25.9 (0.98)	25.7 (0.99)	1.154–1.138	18.8 (0.99)	19.3 (0.99)	1.138–1.170
BW	F	38.8 (0.95)	31.6 (0.90)	1.192–1.142	18.5 (0.98)	20.9 (0.96)	1.142–1.171
Mean		42.0	36.7		15.8	17.1	
±SEM		8.4	8.1		1.2	3.5	
P		<0.005	<0.005		<0.001	<0.005	

[a] The turnover rates (fraction/min) × 10^4 are the slopes obtained by the linear regression analysis of the logarithm of [^{15}N]atom % excess with time.

[b] The range of the ratio of [^{15}N]atom % excess between Plasma (P) and RBC (C) are included to show the trend during that interval.

[c] The numbers in brackets indicate the correlation coefficient ($-r$) obtained in fitting the experimental data by regression analysis.

was very fast and the steady state due to this diffusional flow would have been reached even in the 1 min sample. The subsequent data represented mostly the kinetics of the urea in a different state. A trend of decrease (−0.00021/min) in P/C ratio in the influx experiments and an increase (+0.00013/min) in the efflux experiments with time were seen. Figure 2 also illustrates the change in the P/C ratio from [^{15}N] *in vitro* influx experiments (n = 4). It is to be emphasized that no isotope effects are involved in these studies. The measurement of radioactivity in [^{14}C] experiments reflects the amount of freely exchangeable urea present in the respective compartments whereas in the [^{15}N] studies the atom % excess indicates the relative amount of the tracer to the total urea (sp act). Since the uncertainty in the chemical determination of a small difference in the concentration of urea between plasma and red blood cells will be larger than the difference itself (2), no attempts were made in the [^{14}C] studies to measure separately the urea concentrations in these micro samples. But on the other hand, in the [^{15}N] studies the simultaneous measurement of 28 and 29 mass peak heights leads directly to the sp act [29/2(28+29)].

The possibility for any systematic analytical error in our [^{15}N] studies was also explored. Any contamination of plasma in cells will lead only to a lower P/C ratio and the true value will be still higher than observed. Deproteinizing, permutite and urease treatments, release of ammonia, and tracer analyses were all carried out identically for plasma, cells, and blood. The presence of about 13% more free glutamine (and asparagine) in cells than in plasma (2) may give rise to a slightly different amount of "alkali-labile" ammonia during the Conway diffusion procedure. This may account for a maximum P/C ratio of 1.003 which is negligible when one compares with our mean values of 1.176 and 1.094 from *in vivo* and *in vitro* studies respectively. The low concentration of CO_2-NH_3 adduct (carbamic acid, carbamate, carbamyl phosphate) if any, in cells will not influence our results. Urease blank determinations did not give any detectable (by mass spectrometer) ammonia nitrogen. The absence of urea enzymes in erythrocytes (9) ruled out the possibility of any cell urea production after separation of the two blood phases. A material balance was obtained between the sp act of whole blood and plasma or cells.

A concentration gradient promotes as isotope flux in our *in vitro* studies. The rate of transport of urea from the plasma into red blood cells may be represented by the equation:

$$d\{A\}/dt = -k'\{A\} + k''\{B\} \qquad (1)$$

where A and B are the concentrations of urea in plasma and cells respectively, k' and k'' are the "transfer constants". At equilibrium $d\{A\}/dt$ becomes zero. Thus,

$$k'/k'' = \{B\}_{eq}/\{A\}_{eq} = K \qquad (2)$$

On integrating for a time interval, t_1–t_2, and substituting with respective concentrations (10), equation 1 becomes:

$$-(t_2-t_1)(k' + k'') = ln\,[(\{A\}_2K - \{B\}_2)/(\{A\}_1K - \{B\}_1)]. \qquad (3)$$

Using the measured values of K (0.808 for influx and 0.791 for efflux), the "transfer constants" ($k' + k''$) were calculated for the 0–1 and 1–5 min intervals in the [^{14}C] experiments. The corresponding values (min^{-1}) of ($k' + k''$) were 4.042±0.026 and 0.170±0.008 for the influx studies and 4.548±0.035 and 0.066±0.020 for the efflux studies. In [^{15}N] *in vitro* studies K = 0.907 and ($k' + k''$) for 30–90 min interval was 0.023±0.004.

Discussion. When added to the blood [^{14}C]-labeled urea distributes itself between the cells and plasma in the same ratio of the freely exchangeable urea already present. About 97% of this distribution is attained in less than a minute and it takes about 2–3 hr to complete the equilibrium. Between equal volume of the components [^{14}C] radioactivity counts give directly the distribution ratio of free urea. This ratio at 1 min (1.277±.016, $P < 0.005$) closely follows that of water (1.288±.007, $P < 0.001$) demonstrating the identical concentrations of exchangeable urea per unit volume of either plasma water or cell water. One would expect this identity between these two phases which have almost no

permeability barrier. The analytical techniques followed in other studies (2-5) did not permit to distinguish this similarity in concentration. They have reported that the urea concentration in red cell water is higher than that in plasma water. This type of difference is observed by us in our [^{15}N] experiments where the concentration of the tracer with respect to the total urea (free and bound) was measured. The P/C ratio in our [^{15}N] studies should be one if the urea is present only in the free form. The observed significant deviation of this ratio from unity in both *in vivo* (1.176±.010, Mean±SEM) and *in vitro* (1.094±.011) studies shows clearly that urea is not distributed in the same freely exchangeable form between the blood compartments. The [^{15}N] atom % excess is always higher in plasma indicating that more bound urea is present in cells than in plasma. The P/C ratio is about 7% lower in the case of *in vitro* [^{15}N] studies. This appears to be a significant one since the pooled mean square analysis (11) of the data and the resulted *t*-distribution statistics gives P at <0.005. The small but significant difference may be due to loss of a substrate, similar to that of 2,3-diphosphoglycerate in the binding of oxygen by hemoglobin (12) resulting in less bound urea in cells. Conditions like pH, PCO_2 etc. were not controlled in the *in vitro* studies.

The fractional turnover rate of labeled urea from the plasma is not the same as that from the cells (Table I), indicating a different state of urea. In the initial period (up to about 180 min) there is a fast decay of the isotopic urea and the slope from cells is about 13% less than that from plasma (paired *t*-test $P < 0.02$). The P/C ratio is always greater than one, and there is a trend of its slow decrease with time (Table I). Thus in this period of equilibration there is a slow exchange and replacement of the bound cell urea by the labeled urea. In the second period (180–600 min) the decay of the isotopic urea is slowed down and the turnover rate from cells is about 8% higher than that from plasma. Now the P/C ratio slowly increases with time showing the slow replacement of the labeled bound cell urea. Our value of the fractional turnover rate $(15.8\pm1.2) \times 10^{-4}$ min^{-1}, of urea from the plasma agrees very well with the reported value $(15.5\pm1.1) \times 10^{-4}$ min^{-1} of Walser (13). No studies were done previously with the cells. Whether the [^{15}N] urea is injected (MS-1) or produced endogenously (MS-2), its decay pattern and distribution data between the two components of blood are almost the same indicating the indistinguishability of the source of marker urea.

The trend of decrease with time in the P/C ratio of the radioactivity in *in vitro* influx studies and a trend of increase in P/C ratio of the specific activity in the [^{15}N] *in vitro* experiments (Fig. 2) clearly demonstrate a slow influx of urea into the components of the red blood cells. These *in vitro* results and the absence of parallelism in the rate of clearance of labeled urea from the cells and plasma confirm that urea in human red blood cells exists both in a freely exchangeable form and in a relatively slowly exchangeable bound state. Plasma occupies a central position in the distribution and transport of urea. Both in the hepatic addition and in the renal extraction of urea from the plasma, the concentration in the red cell water is also perturbed but quickly adapted to the changing conditions. The similarity of the transfer constants for urea obtained in the first min of our influx and efflux *in vitro* studies suggests the operation of the same mechanisms in both directions. Following this initial rapid transfer of urea, the observed slowdown may be due only to the interaction with proteins.

Binding of a small molecule by a protein is the pivotal step in a host of biological functions including their transport throughout the vascular system. Significant quantities of urea are known to be bound to various proteins (3, 14). Hemoglobin is the most likely cell constituent that might form a urea complex. There may be a conformational accommodation, which merely stabilizes the complex and does not trigger a physiological process for either the protein or the ligand (15). Actually such a molecular adaptation to the relatively high urea concentration was found *in vivo* by elasmobranch hemoglobins (16).

When the equilibrium is shifted either by addition or extraction of urea, the bound urea becomes labile to maintain equilib-

rium. Although the blood urea nitrogen in renal venous blood is lower than that in the arterial blood by about 9% which corresponds to the urinary excretion, there is no difference between the arterial and venous blood with respect to urea concentrations of red cell water or plasma water (17). This indicates that the perturbed equilibrium between the urea concentration of the red cell water and plasma water due to the overall clearance of urea has already been restored as the blood leaves the kidney. During hemodialysis of uremic patients (18) when the extracellular urea concentration is considerably reduced, it takes about 15 hr post dialysis to attain the predialysis equilibrium between extracellular (plasma) and intracellular (muscle) urea concentrations. It does not take this long to attain equilibrium between red cell water and plasma water (19) but the low degree of hemolysis observed in some cases of dialysis may partly be due to a delay in attaining equilibrium between the bound and free urea especially in red cells.

The distribution ratio of the freely exchangeable urea between equal volumes of plasma and cell is 1.277. The [^{15}N] atom % excess ratio 1.176 (our [^{15}N] *in vivo* mean value) represents the ratio of the ratio between the free urea and total (free and bound) urea present in plasma and cell compartments.

$$F_p/F_c = 1.277 \quad (4)$$

$$(F_p/F_p + B_p)/(F_c/F_c + B_c) = 1.176 \quad (5)$$

Here F and B represent the amount of free and bound urea and the subscripts p and c represent plasma and cells respectively. Thus

$$(F_c + B_c)/(F_p + B_p) = 0.921 \quad (6)$$

Since the cell and plasma proteins have equal binding capacities for urea (3), their respective concentrations 0.3298 g protein/ml cell and 0.07695 g protein/ml plasma may be related to the relative amount of bound urea between the two compartments.

$$B_p/B_c = 0.07695/0.32980 = 0.233 \quad (7)$$

From these relationships one can obtain $F_p/(F_p + B_p)$, $B_p/(F_p + B_p)$, $F_c/(F_c + B_c)$ and $B_c/(F_c + B_c)$ ratios. It is calculated that about 96% urea in the plasma phase is freely exchangeable compared to only 82% in the cells. Thus in normal human blood (Hct. 45) about 10% of the urea is not readily available for free exchange but can be mobilized under specific conditions. In model simulations of urea transfer especially during dialysis (6, 7), this unequal distribution of urea between the two components of blood should be taken into consideration. Neglecting the possible differences between whole blood and plasma concentrations of urea and its rates of equilibration between red cells and plasma, Bass *et al.* (6) found that the calculated clearance of urea is 17% greater than the clearance calculated using whole blood levels and flow, or plasma levels and flow. This suggests the existence of a difference in the rate of removal of urea from the two components of blood which is confirmed by our observations.

Summary. The distribution kinetics of urea between plasma and the red blood cells of normal human blood have been investigated *in vivo* using [^{15}N] urea and *in vitro* using [^{15}N] urea and [^{14}C] urea. The partition ratio of free exchangeable urea between the cell and plasma is found to be the same as that of water distribution, showing the identical concentration (urea/ml water) of free urea in these two compartments of blood. However a significant difference (18%) is observed in the sp act ([^{15}N] atom % excess) between the urea present in the cell and plasma compartments of blood. This indicates that about one-fifth of the cell urea may not be in a freely exchangeable form. The fractional turnover rate of urea from the red blood cells is different from that of the plasma compartment suggesting the relatively bound state of intracellular urea and its slow mobilization.

1. Savitz, D., and Solomon, A. K., J. Gen. Physiol. **58,** 259 (1971).
2. McMenamy, R. H., Lund, C. C., Neville, G. J., and Wallach, D. F. H., J. Clin. Invest. **39,** 1675 (1960).
3. Murdaugh, Jr., H. V., and Doyle, E. M., J. Lab. Clin. Med. **57,** 759 (1961).
4. Ralls, J. O., J. Biol. Chem. **151,** 529 (1943).
5. Van Stone, J., Felts, J., Moore, R., and Nolph, K., Clin. Res. **23,** 508 (A) (1975).

6. Bass, O., Nolph, K. D., and Mahr, J. F., J. Lab. Clin. Med. **86,** 378 (1975).
7. Gibson, T. P., Matsuik, E., Nelson, L. D., and Briggs, W. A., Clin. Pharmacol. Therp. **20,** 720 (1976).
8. Rich, G. T., Sha'afi, R. I., Romualez, A., and Solomon, A. K., J. Gen. Physiol. **52,** 941 (1968).
9. Jones, M. E., Anderson, A. D., Anderson, C., and Hodes, S. Arch. Biochem. Biophys. **95,** 499 (1961).
10. Benson, W. S. "The Foundations of Chemical Kinetics", p. 28, eq. (III 2.8), McGraw-Hill Book Co., Inc., New York (1960).
11. Snedecor, G. W. "Statistical Methods Applied to Experiments in Agriculture and Biology", p. 90, Iowa State University Press, Ames, Iowa, 5th Ed. (1966).
12. Oski, F. A., Sugarman, H., and Miller, L. D., Blood **39,** 522 (1972).
13. Walser, M., and Bodenlos, L. J., J. Clin. Invest. **38,** 1617 (1959).
14. Pasynskii, A. G., and Chernyak, R. S., Doklady Acd. Nauk SSSR **73,** 771 (1950), Chem. Abst. **45,** 685 (1951).
15. Klotz, I. M., Acc. Chem. Res. **7,** 162 (1974).
16. Bonaventura, J., Bonaventura, C., and Sullivan, B., Science **186,** 57 (1974).
17. Van Slyke, D. C., Rhoads, C. P., Hiller, A., and Alving, A. S., Amer. J. Physiol. **109,** 336 (1934).
18. Shackman, R., Chrisholm, G. D., Holden, A. J., and Pigott, R. W., Brit. Med. J. **2,** 355 (1934).
19. Blackmore, D. J., Elder, W. J., and Bowden, C. H., J. Clin. Pathol. **16,** 235 (1963).

Received June 28, 1977. P.S.E.B.M. 1978, Vol. 157.