# PAPER CHROMATOGRAPHY OF SOME ENZYMES AND THE PLASMA PROTEINS*

by

E. CABIB

*Instituto de Investigaciones Bioquímicas, Fundación Campomar, Buenos Aires (Argentina)*

In recent years paper chromatography of proteins has received an increasing attention. FRANKLIN AND QUASTEL[1] and GROSS, LEBLOND, FRANKLIN AND QUASTEL[2], made use of buffer solutions as solvents; but although many proteins were shown to move along the paper, no clear separation between them was obtained. Later, REID[3] was able to separate two enzymes, namely poligalacturonase and pectin esterase from a fungus extract, using FRANKLIN AND QUASTEL's solvents.

TISELIUS[4] has demonstrated that proteins can be adsorbed on filter paper in the presence of various salts in concentration much smaller than that necessary to precipitate them (salting out adsorption). Following this line, MITCHELL *et al.*[5] applied an ammonium sulphate concentration gradient to the filter paper "chromatopile", obtaining a partial separation between the enzymes adenosine deaminase, phosphatase and amylase from a "takadiastase" preparation.

At the same time, methods were developed for the detection of proteins on the chromatogram. FRANKLIN AND QUASTEL[1] added hemin to the protein solution and revealed the spots of protein-hemin complex with the benzidine reagent. DURRUM[6] and CREMER AND TISELIUS[7] stained the protein spots with bromphenol blue, while JONES AND MICHAEL[8] used various dyes, one of them Solvay Purple.

In the investigation presented here the attempt has been made of extending to paper chromatography the use of water-miscible organic solvents for the separation of proteins[9]**.

The first part of this paper deals with the separation of two enzymes—phosphoglucomutase and invertase—from yeast extracts, while in the second part the separation between albumin and the globulins of various sera is described.

## EXPERIMENTAL PART

### *Materials*

*Saccharomyces fragilis* was grown and dried as described by CAPUTTO *et al.*[10]. Palermo brewer's yeast was dried in air at room temperature. Lebedev juice of either yeast was prepared by extraction

* The data in this paper were taken from a thesis submitted in partial fulfilment of the requirements for the degree of Doctor in Chemistry of the Buenos Aires University.

** When this paper was ready to be submitted for publication a report of K. V. GIRI AND A. L. N. PRASAD appeared (*Nature*, 167 (1951) 859) in which the chromatography of some enzymes with aqueous alcohol and acetone is described.

*References p. 614.*

with 3 volumes of 2.2% diammonium phosphate at 5° during 24 hours and dialyzed against water in the cold. In several experiments these solutions were used for chromatography without further purification. In most cases however, a fractionation with ammonium sulphate was carried out, isolating the fraction of the *Saccharomyces fragilis* extract which precipitates between 40 and 65% saturation; for the brewer's yeast juice the limits were 45 and 60% saturation. Either preparation was redissolved in water and dialyzed before use.

Human serum albumin was a crystalline sample and $\gamma$-globulin was electrophoretically homogeneous. A 6% solution of the former in water and a 2% solution of the latter in 0.1 *M* potassium primary phosphate were used for chromatography.

Preparation of the globulins and albumin of rabbit serum was accomplished by precipitation with ammonium sulphate at 50% and between 50 and 75% saturation, respectively. Both fractions were purified by several reprecipitations. Finally albumin was dissolved in water and dialyzed against water, while the globulins were dissolved in dilute phosphate solution and dialyzed against phosphate.

SÖRENSEN phosphate buffers were used throughout in the solvent mixtures, except for the buffer of pH 4.4, which was a 0.1 *M* acetic acid-acetate solution. The maleate buffer was prepared according to SMITS[11].

The concentration of ethyl alcohol in the solvents will be given in ml of 100% alcohol per 100 ml of solution.

Glucose-1-phosphate was prepared according to SUMNER AND SOMERS[12]. Synthetic glucose-1,6-diphosphate was obtained by the method of LELOIR *et al.*[13].

The sugars used were all commercial samples.

## *Methods*

### *Chromatography*

The ascending method of HORNE AND POLLARD[14] and WILLIAMS AND KIRBY[15] was employed in most runs. Vessels were one and two litres graduated cylinders, closed with a rubber sheet, tightened with an elastic band.

Whatman No. 1 filter paper was used throughout. Strips 36 cm long and 4 or 7.5 cm wide were cut and the starting line was ruled 5 cm from one end. In the chromatography of enzymatic extracts, and in some experiments with plasma proteins, one band of the solution about 1 cm wide was applied to the paper. In most runs with plasma proteins, however, 5 $\mu$l drops were placed with a capillary pipette, at a distance of 2 cm one from the other and from the edge of the strip. Chromatography was started immediately, without previous drying of the solutions, in order to lessen the denaturation of proteins. The solvent was allowed to travel 20–25 cm from the origin.

### *Enzymatic activities determinations*

After chromatography the paper was hung at room temperature, whereby a partial evaporation of the solvent took place. Then the paper, still moist, was divided with scissors into longitudinal strips 1.9 cm wide. Each strip was cut transversally at 3 cm intervals, and the 1.9 × 3 cm pieces obtained were placed in test tubes to which the substrate was added. A paper blank for each enzyme determination was run at the same time. All the tubes were incubated at 37° during 40 min, whereupon the contents were analyzed without removing the papers. Thus, in certain cases as many as three different enzymes could be assayed simultaneously, using one paper strip 7.5 cm wide. The enzymatic activities were then plotted as a function of the paper length.

The activity measurements for the different enzymes were conducted as follows:

*Phosphoglucomutase.* Essentially as described by CARDINI *et al.*[16]. The reaction mixture was: glucose-1-phosphate, 2 $\mu$M; glucose-1,6-diphosphate, $1 \cdot 10^{-5}$ $\mu$M; magnesium sulphate, 2 $\mu$M, histidine, 1.2 $\mu$M[17]; total volume, 0.6 ml. In some cases histidine was substituted by 0.01 *M* potassium cyanide (final concentration). When the enzyme was placed on paper about half the activity was lost, but it was partially restored upon addition of histidine or cyanide.

*Invertase.* Substrate: 1 $\mu$M sucrose in 0.6 ml water. The reaction was stopped by addition of 1.5 ml of the Somogyi copper reagent[18]. The reducing power was determined as for phosphoglucomutase[16]. When raffinose was replaced for sucrose, 6 $\mu$M of that sugar were added to each tube and the incubation time was 2 hours.

*$\alpha$-Glucosidase.* Substrate: 1 $\mu$M of maltose or $\alpha$-methyl glucoside in 0.6 ml water. When maltose was the substrate, the liberated glucose was determined with the Barfoed reagent as modified by CAPUTTO *et al.*[10].

### *Detection of proteins on the chromatograms*

The method given by DURRUM[6] with bromphenol blue in mercuric chloride saturated alcoholic solution was used throughout. In some instances, however, the paper was cut into transversal strips 2 cm wide, each strip extracted with water, and the optical density at 280 m$\mu$ read in the Beckman spectrophotometer.

*References p. 614.*

## RESULTS

### *Chromatography of yeast extracts*

Some experiments were carried out applying to the paper an ammonium sulphate concentration gradient, according to MITCHELL *et al.*[5], but it was found that the enzymes were spread over a large zone of the paper strip. Also, the inhibition of the activities by the high salt concentration interfered with the enzymatic assay. Moreover, determination of the ammonium sulphate concentration along the paper strip did not give a steadily rising curve from the origin up to the solvent front; the salt concentration rised from the starting line to one point placed at about the middle of the paper and then

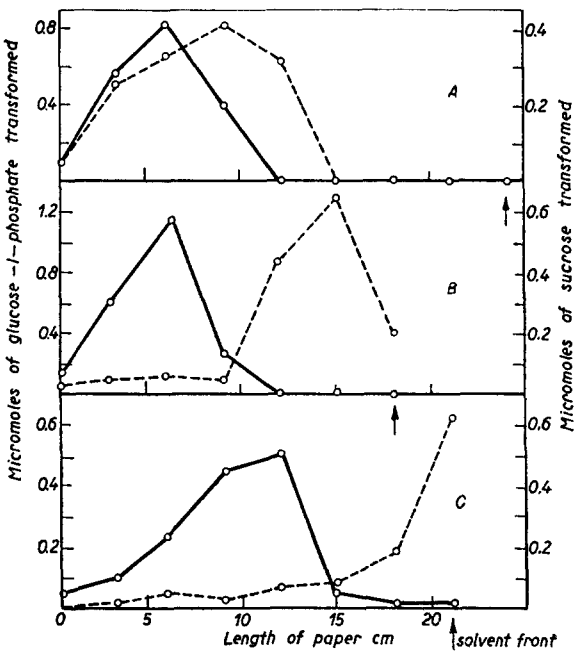


Fig. 1. Chromatography of a purified extract from *Saccharomyces fragilis* with ethanol-phosphate buffer mixtures. Full line, phosphoglucomutase activity. Broken line, invertase activity. Concentration of ethanol in the solvent: A, 30%; B, 25%; C, 20%.

dropped rapidly. Similar findings, although without using a concentration gradient, have been reported by HORNE AND POLLARD[14] and more recently by REID[3].

The poor results obtained with the method described, stimulated the search for other solvents: thus mixtures of ethyl alcohol with phosphate buffers at pH 7 were tried. In these experiments paper was also impregnated with buffer[19]. Runs were carried out at low temperature to avoid as much as possible inactivation of enzymes.

The enzyme solution submitted to chromatography was a partially purified *Saccharomyces fragilis* extract. The enzymes assayed were phosphoglucomutase and invertase. Using appropiate alcohol concentrations a thorough separation of the two enzymes was attained, as shown in Fig. 1, where the effect of changing the alcohol proportion in the solvent mixture is also demonstrated.

It is apparent that invertase moved faster as the alcohol concentration decreased,

*References p. 614.*

while the effect was not so pronounced with phosphoglucomutase. At concentrations lower than 25 per cent. alcohol the latter enzyme spread over a large portion of the paper.

When the chromatography was repeated using an extract from brewer's yeast, obtained in a similar way as that of *Saccharomyces fragilis*, a complication appeared. While phosphoglucomutase behaved as in the former case, two peaks of invertase activity were found (see Fig. 2). The possibility that sucrose might be split in the two peaks by different enzymes, for instance a β-fructosidase and an α-glucosidase[20], was investigated using other substrates. The extract used for chromatography contained enzymes able to hydrolyze maltose or α-methyl glucoside. However, when activity of such enzymes was tested in the paper strip, after chromatography, none was encountered, as if they had undergone complete denaturation. In these experiments the solvent mixture was made up with maleate buffer instead of phosphate, as the latter interfered with the determination of maltose. Previously it was verified that an analogous distribution of invertase-like activity along the paper strip was obtained with either phosphate or maleate

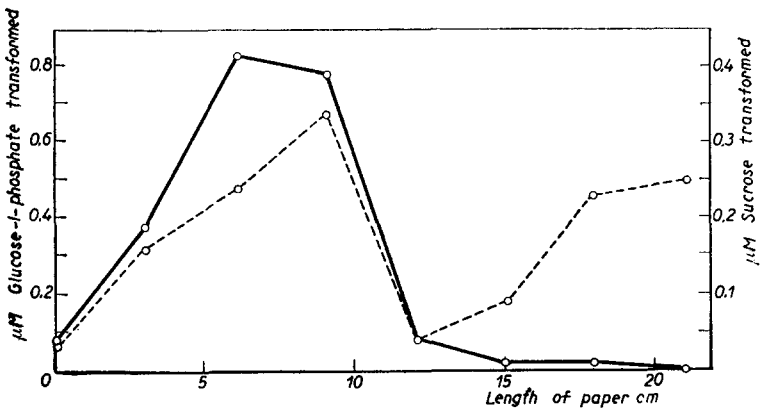


Fig. 2. Chromatography of a purified extract from brewer's yeast. Solvent: phosphate buffer of pH 7 with 25% ethanol. Full line, phosphoglucomutase activity. Broken line, invertase activity.

in the solvent. This apparently rules out with the possibility of a sucrose phosphorylase activity; moreover, an experiment in which the solvent contained maleate buffer and activity was tested with and without addition of phosphate to the reaction mixture, gave an identical distribution pattern in either case.

The two peaks of activity were also obtained when raffinose, the β-fructoside of melibiose, was tested as substrate after chromatography.

Finally, the enzyme extracted from each of the two zones of maximum activity was allowed to act on sucrose, and the resulting solution, after evaporation, was submitted to paper chromatography using the ethyl acetate-pyridine-water solvent of JERMYN AND ISHERWOOD[21]. In both cases the reaction products were identified as glucose and fructose by their $R_F$ and the colour developed with the aniline phthalate[22] and the resorcinol reagents[23].

From the experimental evidence now available it may thus be concluded that the two enzymatic activities are very similar, if not identical, as to substrate specificity and the reaction catalyzed.

*References p. 614.*

*Chromatography of the plasma proteins*

Chromatograms of human serum crystalline albumin using ethanol-water mixtures as solvents, and bromphenol blue[6] as the colour reagent showed usually a single spot. The $R_F$ varied from 0.86 to 0.60 as the ethanol concentration was raised from 10 to 40 per cent. With 40 per cent. ethanol an additional spot was observed of $R_F$ 0.10. Human plasma and horse, rabbit and human serum gave more or less extended streaks starting from the origin, and spots with $R_F$ similar to but slightly lower than that of crystalline albumin, as can be seen in Fig. 3 and in Table I.

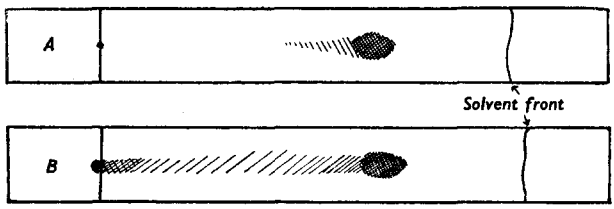


Fig. 3. Chromatogram of the serum proteins developed with 30% aqueous ethanol. A, crystalline human serum albumin. $R_F$, 0.68. B, human serum. $R_F$ of the albumin spot, 0.67.

TABLE I

$R_F$ OF ALBUMIN WITH ETHANOL-WATER AND ETHANOL-BUFFER MIXTURES

| *Ethanol Concentration* | *Sample submitted to chromatography* | $R_F$ | |
|---|---|---|---|
| | | *Ethanol-water* | *Ethanol-buffer* |
| Per cent. v/v | | | |
| 10 | Crystalline albumin | 0.86 | 0.91 |
| 10 | Human serum | 0.80 | 0.88 |
| 10 | Horse plasma | — | 0.81 |
| 20 | Crystalline albumin | 0.81 | 0.90 |
| 20 | Human serum | 0.75 | 0.78 |
| 20 | Horse plasma | — | 0.83 |
| 30 | Crystalline albumin | 0.68 | — |
| 30 | Human serum | 0.67 | — |
| 40 | Crystalline albumin | 0.60 | — |
| 40 | Human serum | 0.45 | — |

The albumin and globulin fractions of rabbit serum were separated by ammonium sulphate fractionation and run simultaneously with the untreated serum, using 30 per cent. ethanol as solvent. One of these chromatograms is shown in Fig. 4. It is apparent that the streak obtained with plasma and serum was due to the globulins, and the fast running spot to albumin. Acetone-water mixtures (30 per cent. aqueous acetone)

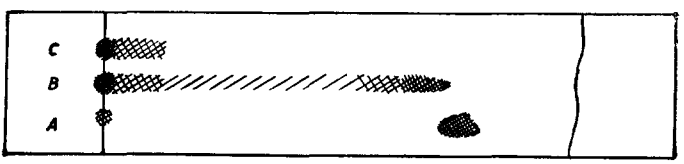


Fig. 4. Chromatogram of rabbit serum and the albumin and globulin fractions, developed with 30% aqueous ethanol. A, albumin fraction. B, whole serum; C, globulin fraction.

*References p. 614.*

yielded chromatographic patterns similar to the above described, except for globulins, which showed a slowly moving definite spot inside the elongated streak.

The effect was studied next of replacing water in the solvent with buffer solutions. In the first series of experiments the pH of the buffer was held constant at 6.5, while the ethanol concentration was varied from 10 to 40 per cent. Spots and streaks were, as a rule, more elongated than with alcohol-water mixtures. Furthermore, at low ethanol concentration (10 to 20 per cent.) a separation between albumin and globulins was obtained, but at higher concentrations (30 to 40 per cent.) only a long streak was observed with any of the protein mixtures assayed. For the $R_F$ of the albumin spot see Table I. In the second series runs were carried out at four different pH values (4.4, 5.3, 6.5 and 8), while the ethanol concentration remained fixed at 20 per cent. It has been mentioned previously that at pH 6.5 with that alcohol concentration in the solvent, the results did not differ very much from those obtained with the analogous ethanol-water mixtures. This was also the case at pH 5.3 and 8, but not at pH 4.4. Here human serum, horse plasma and crystalline albumin gave long streaks without separation, while rabbit serum showed two components for the albumin fraction (Fig. 5). With some of the solvents described runs with an electrophoretically pure $\gamma$-globulin were made; it generally behaved as the globulin fraction of rabbit serum.

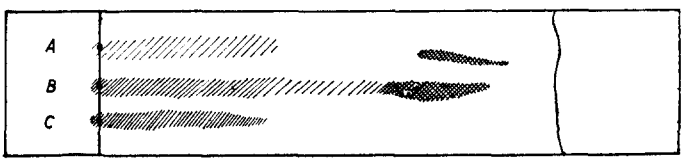


Fig. 5. Chromatogram of rabbit serum and the albumin and globulin fractions, developed with a mixture of acetic acid-sodium acetate buffer of pH 4.4 with 20% ethanol. A, albumin fraction; B, whole serum; C, globulin fraction.

Buffers (without alcohol) gave no separate spots with plasma and sera. Slightly better results were obtained with ammonium sulphate solutions, particularly when an ammonium sulphate gradient was applied to the paper, but the marked "tailing" noticed with these solvents discouraged further experimentation along this line.

At this point it should be reminded that in any of the chromatograms, no matter the solvent used, a "tailing" effect was observed behind the albumin spot; the tail was much more pronounced with plasma or a mixture of the rabbit serum albumin and globulins fractions, than with albumin alone. Moreover, not only the $R_F$ of albumin was lower in the presence of globulins, but it was progressively diminished as the globulins proportion in the mixture was raised, as shown in Fig. 6.

In several experiments a band, instead of a drop, of the protein solution submitted to chromatography was applied at the starting line. With solvents containing more than

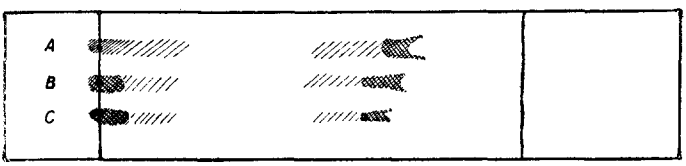


Fig. 6. Chromatogram of different mixtures of the albumin and globulin fractions of rabbit serum. A: 3 parts of albumin with 1 part of globulin. B: equal amounts of the two fractions. C: 1 part of albumin with 3 parts of globulin. The $R_F$ values of the albumin spot are respectively 0.69, 0.65, 0.62.

*References p. 614.*

20 per cent. of ethyl alcohol the resulting band of albumin in the developed chromatogram was fairly uniform, but with more dilute solvents very irregular bands, often divided in sectors, were obtained. These results are to be related to the resistance opposed by proteins to the solvent flow. The solvent does not diffuse across the band in a homogeneous manner, but rather it penetrates it at certain sites; later it diffuses laterally to fill all the width of the strip, and so distributes unevenly the proteic material. This effect is more marked with dilute, low-viscosity solvents, which diffuse rapidly across the paper.

## DISCUSSION

The alcohol-buffer mixtures have led to a good separation between two enzymes, a serious drawback of the method should nevertheless be considered: this is the spreading of each active protein in a very diffuse band, at least 6 to 7 cm wide, for a total distance travelled by the solvent of about 25 cm.

The case of the brewer's yeast invertase seems to point to a heterogeneity of the enzyme molecules; while perhaps the available data are not sufficient to warrant the absolute identity of the enzymatic activities on the two peaks, it may be of interest to mention that REID has encountered a similar effect with a fungus poligalacturonase[3].

Many of the solvent assayed yielded an excellent separation between albumin and the globulins of plasma. On the other hand it has not been possible to obtain more than a single definite spot (or streak) from the globulin fraction.

Some facts point to an interaction between albumin and the globulins; these are the pronounced tailing and the lower $R_F$ of the albumin spot in the presence of globulins. As previously mentioned, the latter effect was greater as the globulins proportion in the mixture increased.

The phenomena observed when a band, instead of one drop of solution was applied to the paper, may account for some results obtained by FRANKLIN AND QUASTEL[24], when applying bidimensional paper chromatography to plasma. In the chromatograms presented by these authors a great number of elongated spots can be seen along the solvent front in the second direction of running. Actually, we have obtained a chromatogram apparently similar to those of FRANKLIN AND QUASTEL in the following way: a band of serum was extended along one side of a filter paper sheet, and a 0.1 $N$ tartrate solution was allowed to run in a direction perpendicular to the starting line; the result is shown in Fig. 7. This demonstrates that many spots may be obtained even if no separation in the first dimension had been effected.

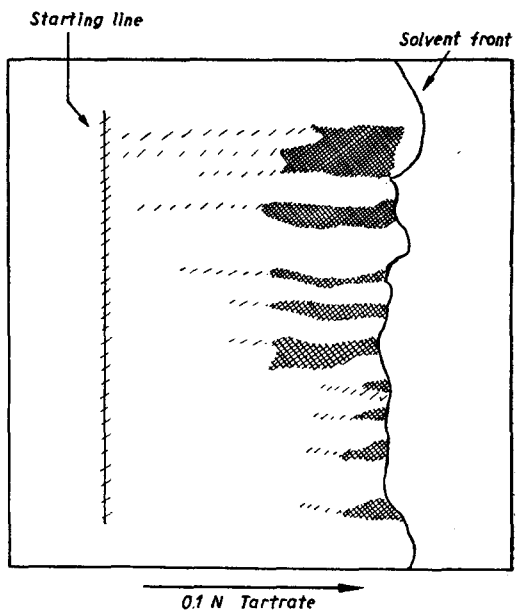


Fig. 7. Chromatogram of rabbit serum developed with 0.1 $N$ sodium-potassium tartrate

*References p. 614.*

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr L. F. LELOIR for his help and encouragement, and to Drs R. CAPUTTO, C. E. CARDINI and A. C. PALADINI for useful suggestions and criticism. I am also indebted to Dr N. MITTELMAN for a generous gift of crystalline serum albumin and $\gamma$-globulin and to Dr A. PARODI for several samples of sera.

SUMMARY

Two enzymes, *i.e.* phosphoglucomutase and invertase, from a *Sacch. fragilis* extract, have been separated by paper chromatography, using ethanol-buffer mixtures as solvents. Under the same conditions invertase from brewer's yeast gave two peaks of activity.

Separation between albumin and the globulin fraction of various sera has been carried out with ethanol-water and ethanol-buffer mixtures. The effect of changing the alcohol concentration and the pH of the solvent was investigated.

RÉSUMÉ

On a séparé deux enzymes, la phosphoglucomutase et l'invertase d'un extrait de *Sacch. fragilis* à l'aide de la chromatographie sur papier, utilisant comme phases solvantes des mélanges d'éthanol avec des tampons. D'autre part, dans ces conditions l'invertase de levure de bière montre deux maxima d'activité.

L'albumine et la fraction globulinique de plusieurs sérums ont été séparées par des mélanges éthanol-eau et éthanol-tampon. On a étudié l'influence de la concentration d'alcool et du pH sur ces séparations.

ZUSAMMENFASSUNG

Man hat zwei Fermente, die Phosphoglukomutase und die Invertase, aus einem Extract von *Sacch. fragilis* durch Papierchromatographie getrennt. Dafür benützte man als Lösungsmittel Äthanol-Puffer Mischungen. Unter diesen Bedingungen, zeigt die Invertase der Bierhefe zwei Aktivitätsmaxima.

Das Albumin und die Globulinfraktion von verschiedenen Sera wurden durch Äthanol-Wasser und Äthanol-Puffer Gemischen getrennt. Man hat auch die Wirkung der Konzentrationen von Alkohol und die Wirkung des pH auf diese Trennungen studiert.

REFERENCES

1 A. E. FRANKLIN AND J. H. QUASTEL, *Science*, 110 (1949) 447.
2 J. GROSS, C. P. LEBLOND, A. E. FRANKLIN, AND J. H. QUASTEL, *Science*, 111 (1950) 605.
3 W. W. REID, *Nature*, 166 (1950) 569.
4 A. TISELIUS, *Arkiv Kemi Mineral. Geol.*, 26B (1948) 1.
5 H. K. MITCHELL, M. GORDON, AND F. A. HASKINS, *J. Biol. Chem.*, 180 (1949) 1071.
6 E. L. DURRUM, *J. Am. Chem. Soc.*, 72 (1950) 2943.
7 H. D. CREMER AND A. TISELIUS, *Biochem. Z.*, 320 (1950) 273.
8 J. I. M. JONES AND S. E. MICHAEL, *Nature*, 165 (1950) 685.
9 J. T. EDSALL, *Advances in Protein Chem.*, 4 (1947) 384.
10 R. CAPUTTO, L. F. LELOIR, AND R. E. TRUCCO, *Enzymologia*, 12 (1948) 350.
11 G. SMITS, *Biochim. Biophys. Acta*, 1 (1947) 280.
12 J. B. SUMNER AND G. F. SOMERS, *Arch. Biochem.*, 4, (1944) 11.
13 L. F. LELOIR, O. M. REPETTO, C. E. CARDINI, A. C. PALADINI, AND R. CAPUTTO, *Anales Asoc. Quím. Argentina*, 37 (1949) 187.
14 R. E. HORNE AND A. L. J. POLLARD, *J. Bacteriol.*, 55 (1948) 231.
15 R. J. WILLIAMS AND H. KIRBY, *Science*, 107 (1948) 481.
16 C. E. CARDINI, A. C. PALADINI, R. CAPUTTO, L. F. LELOIR, AND R. TRUCCO, *Arch. Biochem.*, 22 (1949) 87.
17 E. W. SUTHERLAND, *J. Biol. Chem.*, 180 (1949) 1279.
18 M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 61.
19 S. BLACKBURN AND A. G. LOWTHER, *Biochem. J.*, 48 (1951) 126.
20 R. WEIDENHAGEN, in F. F. NORD AND R. WEIDENHAGEN, *Handbuch der Enzymologie*, Leipzig (1940) 514.
21 M. A. JERMYN AND F. A. ISHERWOOD, *Biochem. J.*, 44 (1949) 402.
22 S. M. PARTRIDGE, *Nature*, 164 (1949) 443.
23 W. G. C. FORSYTH, *Nature*, 161 (1948) 239.
24 A. E. FRANKLIN AND J. H. QUASTEL, *Proc. Soc. Exptl. Biol. Med.*, 74 (1950) 803.

Received August 13th, 1951