PAPYROGRAPHIC* STUDIES IN NITROGEN METABOLISM OF MICRO-ORGANISMS

Part I. A Critical Study of the One-Dimensional Micromethod of Papyrography for the Analysis of Protein Hydrolysates

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IN the course of our studies on the nitrogen metabolism of micro-organisms in relation to their mitotic cycle, we were faced with the problem of partitioning the nitrogenous constituents of the cell. Two other studies which presented the same problem were, (1) the amino acid make up of the malarial parasite and (2) the biological efficiency of the silkworm as a converter of the feed protein into silk protein.

The choice of the method would naturally be influenced by its simplicity, speed and ability to deal with micro quantities of the research material. Papyrography originated by Consdon, *et al.*,[1] offers a suitable method and meets most of the requirements. But, in view of the acute shortage of the essential solvents and developing reagents in this country, we have been obliged to prefer the one dimensional micro-modification of Rockland and Dunn,[2] which has been shown to be suitable for the analysis of amino acids in microgram quantities.

It was of interest to examine if this method could be extended for the detection, separation and estimation of amino acids in protein hydrolysates. The scheme entailed a study of (1) the choice of more effective solvents securing better resolutions of the mixture, (2) the standardisation of the experimental conditions, e.g., quantity of mixture under test, pH, temperature and time of experimentation, (3) conditions for development of colour with ninhydrin and (4) the influence of polypetides, sugars and other interfering substances associated with hydrolysates of tissues and tissue fluids.

Experimental.—Test tubes 6" × ½" with 0·5 ml. of the solvent mixture for developing the chromatogram and filter-paper (Whatmann No. 1) strips measuring 135 mm.×15 mm. tapering to 10 mm. assembled as in Fig. 1 were used for all the experiments. Later for obtaining better resolutions of the mixture, flat bottomed test tubes 8" × 1", 1 ml. developing solvent mixture, and filter-paper strips 180 mm. × 20 mm. tapering down to 15 mm. were used. Solvent mixtures employed consisted of (1) phenol saturated with water,[1] (2) *n*-butanol saturated with water and (3) *n*-butanol saturated with aqueous acetic acid.[3]

The colour is developed with ninhydrin by spraying a solution of the reagent (0·1 per cent.) in *n*-butanol on the filter paper strip after developing the chromotogram.

Different mixtures of pure amino acids, 0·01M solutions and casein hydrolysate, 5-10 mgm. nitrogen/ml. have been used in the course of these studies. 0·5 to 1·0μl of the solution is delivered by means of capillary pipette at a previously marked spot (see Fig. 1). Care is taken to secure

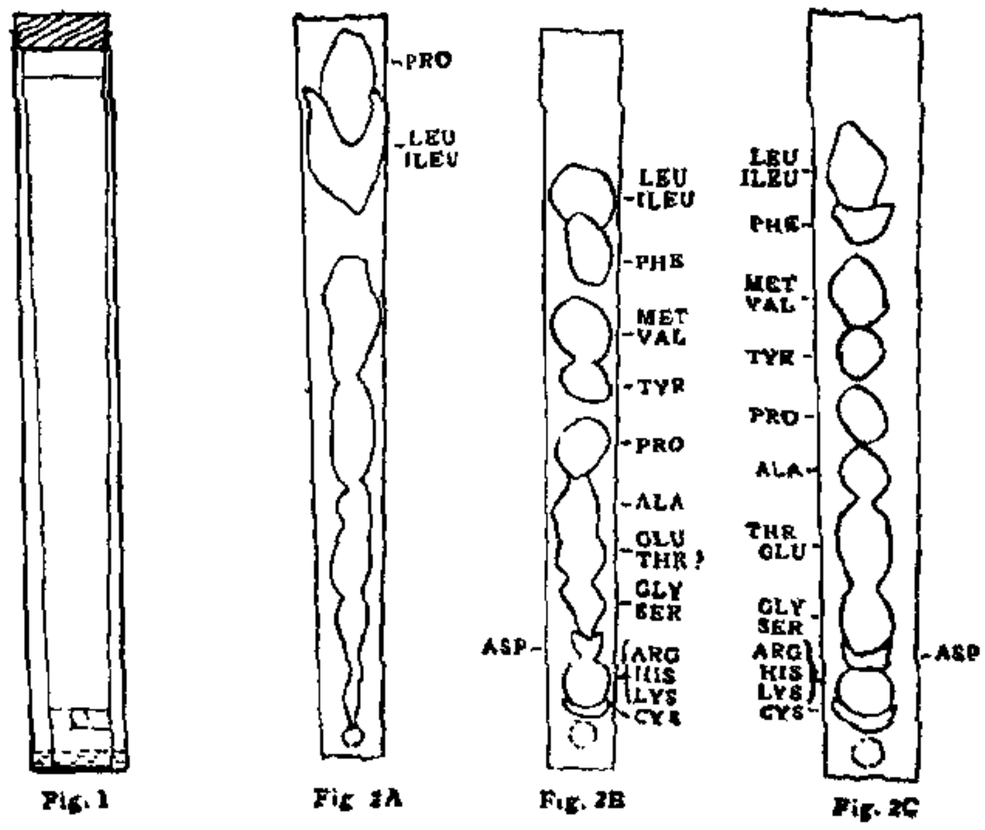

Fig. 1 Fig. 2A Fig. 2B Fig. 2C

FIG. 1 Diagram showing strip in position for developing. The strip is fixed to the cork stopper by a pin. The solvent travels upto the line near the top.

FIG. 2 A.—Reproduction of Papyrogam of Casein (Acid) hydrolysate developed with phenol in small strip.

FIG. 2 B.—Reproduction of Papyrogram of Casein (Acid) hydrolysate developed with *n*-butanol / acetic acid in small strip.

FIG. 2 C.—Reproduction of Papyrogram of Casein (Acid) hydrolysate developed with *n*-butanol / acetic acid in longer strip.

(Abbreviations according to Brand and Edsall, [11])

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* "Papyrography," a suggestive and appropriate term proposed by Dent[6] for partition chromatography on filter-paper is used throughout this paper. Also the word "Papyrogram" is used to denote the map after developing with ninhydrin or other reagents.

a clean circular spot with a diameter not exceeding 2 mm. If the solution happens to be too dilute, the application of the solution at the spot may be repeated after drying out the previously applied solution.

An ascending distance of 125 mm. in the case of the small test tube and a distance of 160 mm. in the case of the bigger test tube are marked and the developing solvent generally takes about 2·5 hours and 4 hours respectively to attain these heights. After development of the column the strips are air dried, sprayed on either side with 0·1% solution of ninhydrin in *n*-butanol and oven dried at 100° C. for 10 minutes with a view to develop the colour. The strip is then viewed both by transmitted and reflected lights and the coloured areas marked with a pencil.

The R_F values[1] of individual amino acids both for phenol and for *n*-butanol/acetic acid were determined when run as single amino acids and also when present as simple mixtures and in protein hydrolysates. The R_F values of a few simple dipeptides have also been determined.

Our experience with phenol has not been very satisfactory for the separation of amino acids. The solvent is corrosive; the spots become diffuse and merge into one another.

n-Butanol saturated with water is found very unsatisfactory since the movement of amino acids was found to be very slow.

n-Butanol saturated with aqueous acetic acid[3] gives a satisfactory separation with a complex mixture of amino acids. The spots, while some of them certainly represent composite spots of a group of closely related amino acids, were discrete and sharply defined.

With 180 mm. strip, better results were obtained.

Discussion.—Phenol, collidine and such solvents have been reported[4,5] previously to decompose partially or fully some of the amino acids. As a result of this the spots spread out, a typical example being cystine giving an elongated spot with R_F values 0.25 to 0.5 Dent[6] has suggested the oxidation of cystine to cysteic acid by H_2O_2 before development. Eesides the sensitivity of the ninhydrin reaction for some amino acids also decreases.[8] Alcohols being comparatively inert, they may be expected to cause no decomposition and this is borne out by our experience.

The R_F values of amino acids are not constant and cannot be relied upon for the identification.[8,9] Various factors influence the movement of an amino acid in relation to the solvent, *e.g.*, slight change in the quality of the solvent

TABLE I

R_F Values of individual Amino Acids

Amino Acids	Solvent : Phenol		Solvent : *n*-Butanol/Acetic acid		
	Authors	Rockland & Dunn[2]	Authors		Woiwod[3*]
			Small strip	Long strip	
Alanine ..	0·62	0·62	0·34	0·35	0·32
Arginine ..	..	0·53	..	0·14	0·11
Aspartic Acid ..	0·05	0·25	0·14	0·19	0·14
Cystine ..	0·25–0·5	..	0·06	0·08	0·03
Glutamic Acid ..	..	0·39	0·45	0·26	0·25
Glycine ..	0·48	0·49	0·34	0·2	0·19
Histidine ..	..	0 81	..	0·11	0·11
Isoleucine ..	0·89	0·85	0·72	..	0·85
Leucine ..	0·88	0·86	0·69	0·66	0·85
Lysine ..	..	0·41	..	0·12	0·11
Methionine ..	0·82	0·74	0·62	0·48	0·62
Phenylalanine ..	0·83	0·87	0·68	0·6	0·76
Proline ..	0·89	0·87	0·41	0·4	0·4
Serine ..	..	0·33	..	0·19	0·19
Threonine ..	..	0·57	..	..	0·25
Tryptophane ..	0·8	0·81	0·64	0·53	..
Tyrosine ..	0·61	0·53	0·5	..	0·53
Valine ..	..	0·82	..	0·47	0·62

* Calculated from reproduction of Papyrogram of mixture of Amino acids taking R_F value of proline to be same as ours.

or the associated constituent in the mixture, the quality of the paper, the degree of saturation of water in the mobile phase, etc. Many of these conditions of experiments can be controlled but occassionally, erratic R_F values were obtained.

It is fortunate, however, that the relative positions of various amino acids are in the same order, irrespective of variations of the individual R_F values. So with a known mixture of some amino acids as reference it should be possible to identify the constituents of an unknown mixture, when run simultaneously. Proline, because of its yellow colour and phenylalanine, because of the bluish purple colour with ninhydrin, constitute convenient reference points.

Martin[10] in a paper to the symposium on Chromatography observes that "in two dimensional chromatograms 1 to 2 micrograms of amino acids could be detected and in single dimensional chromatograms half a microgram can, under favourable circumstances, be observed". From a study of the sensitivity of ninhydrin reaction in papyrography, Pratt and Auclair[8] finds that 10 of the amino acids studied could be detected in microgram or less quantities, in a two-dimensional run with phenol and collidine. The sensitivity of the test is affected by the spreading of spot that occurs in two dimensional run over long hours and possible decomposition by developing solvents. Hence it could be expected that a short run and less reactive solvents would give a compact spot and increase sensitivity of the ninhydrin reaction. As in all micromethods, the quantity of the mixture to be used should be small, to avoid overloading and freakish development. We have been able to confirm the findings of Rockland and Dunn[2] that microgram quantities of amino acids could be detected in the microadaptation of the method. Besides we have found that the use of *n*-butanol acetic acid gives a more compact spot, thus aiding better separations and detections in an analysis of mixtures.

Attempts have been made by some [6,7,11,12] to use other reagents giving colour with specific amino acids. We have also used the Sakaguchi reagent for arginine, Pauly reagent for histidine, Ehrlich reagent for tryptopene and tyrosinase for tyrosene on filter paper strips after development and found them capable of testing microgram quantities. Other specific colour reactions are being tried.

The use of impregnated paper [13,14] is another development which has proved useful in specific cases. We are experimenting with papers impregnated with starch, silica gel, etc Preliminary results give promise of better development on starch impregnated papers in the form of sharp or compact spots though no difference could be found in separation.

Little work has been done on peptides separation,[15,16] by papyrography. Available peptides have been studied in the 180 mm. strip and the results obtained with leucine, glycine and the dipeptides composed of these two are given below (See Table).

R_F *Values of Glycyl-leucine and Leucyl-glycine*

	Phenol		*n*-Butanol/ Acetic acid
	Authors Consdon, *et al.* *[15]		
Glycyl-leucine	0·80	0·87	0·65
Leucyl-glycine	0·74	0·86	0·63
Leucine	0 88	0·88	0·66

* Phenol with 0·1% Cupron and by descending boundary method.

With phenol, the leucylglycine occupies the lower half of an elongated spot, while leucine and glycyl leucine occupy the upper half of the spot. With butanol-acetic acid, leucylglycine and glycyl-leucine occupies the lower half of the leucine spot. The two peptides give a brown colour first[15,16] and so is discernible from leucine in mixed spots. The colour of the dipetides spot however changes to purple slowly.

Summary.—A critical study of the microadaptation of papyrography has been made and improvements in the use of *n*-butanol/acetic acid as developing solvent and use of a slightly longer strip have been suggested. These improvements increase the sensitivity of the technique due to minimum decomposition and spreading of amino acids. The modified technique has been adopted for a routine qualitative analysis of protein hydrolysates.

Possibilities of extending and improving this technique by use of specific colour reactions for some of the amino acids and by use of impregnated papers with a view to secure better separations are discussed.

We wish to express our grateful thanks to the Council of Scientific and Industrial Research for financing a scheme of which this work forms a part. Our thanks are also due to the Director, Indian Institute of Science, for his kind interest.

1 Consdon, R., Gordon, A. H. and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224. 2. Rockland, Louis, B., and Dunn, Max S., *Science*, 1949, **109**, 539 3. Woiwod, A. J., *J. Gen. Microbiol.*, 1949, **3**, 312. 4. Miettinen, J. K. and Virtanen, Artturi I., *Acta Chem. Scand.*, 1949, **3**, 459. 5. Martin, A. J. P., and Mittelman, R., *Biochem. J.*, 1948, **43**, 353. 6. Dent, C. E., *Ibid.*, 1947, **41**, 240.

7. —, *Ibid.*, 1948, **43**, 169. 8. Pratt, John J., and Auclair, Jacques, L., *Science*, 1948, **108**, 213. 9. de Verdier, Carl-Henric and Agren, Gunner, *Acta. Chem. Scand.*, 1948, **2**, 783. 10. Martin, A. J. P., *Ann. N. Y. Acad. Sci.*, 1948, **49**, 259. 11. Agren, Gunner and Nilsson, Tage, *Acta. Chem. Scand.*, 1949, **3**, 525. 12. Dent, C. E. and Rose, G. A., *Biochem. J.*, 1949, **44**, 612. 13. Hopf, P. P., *J. Chem. Soc.*, 1946, 785. 14. Datta, S. P., Overell, B. G., and Stack-Dunne, *Nature*, 1944, **164**, 673. 15. Consdon, R., Gordon, A. H., Martin, A. J. P., *Biochem. J.*, 1947, **41**, 590. 16. —, *Ibid.*, 1949, **44**, 548. 17. Brand, E., and Edsall, J. T., *Ann. Rev. Biochem.*, 1947, **16**, 223.

SCIENCE AND COMMONSENSE*

COMMONSENSE is no doubt a highly valued body of knowledge doing excellent service in the ordinary day-to-day affairs of life, but it cannot be claimed that it is in anyway infallible. One might say that the progress of science and mathematics has to a large extent depended on the discovery of the vast regions where the laws born of common experience are no longer valid, as also through the unearthing of the many pitfalls in the process of deductive reasoning on which commonsense generally depends.

There are many laws relating to the motion of terrestrial objects which are quite true in the immediate range and vicinity where we live and these are embodied in the Newtonian mechanics. But these laws break down when they are carried over to the region of very small bodies such as the electron, whose dimensions are of the order of 10^{-12} cm. (that of our own being 10^2 cm. or so) as well as in the region of the interstellar spaces where the order of reckoning is 10^{12} cm. or thereabouts. Thus, while it is possible to define the position and velocity of a terrestrial object such as an aeroplane with sufficient precision so as to be able to hit it, we are not able to do the same in the case of an object such as the electron without introducing an element of uncertainty.

Similarly, in the field of mathematics, there are many examples where the commonsense view comprehends only a very limited domain of the entire body of truth. For instance, the ordinary commutative law: $a \times b = b \times a$, on which commensense algebra rests, is not universally true, and thus has to make room for the noncommutative algebra, wherein $a \times b$ is not equal to $b \times a$, but quite different.

Also, in the case of velocities approaching to that of light, the commonsense aspect has been found to be so inadequate indeed as to have given rise to the Theory of Relativity. According to this theory, the relative velocity of two particles approaching each other, of which the velocity of the one is u and that of the other is v, is not their sum but may be considerably different, depending on how near their velocities are to that of light.

One more instance where the validity of common experience breaks down is in the province of temperature, where it is well known that all our sensations of life are limited to a few degrees this way and that of the freezing point of ice. But recent experience has shown that below this range, phenomena take place which need for their comprehension and explanation a modification if not also the abandonment of all commonsense ideas referring to conductivity, resistance, fluidity and so on. To illustrate: We all know that a loop of wire in which an electrict current has somehow been introduced soon loses it, by reason of its electrical resistance; but, if the same loop be lowered into a bath whose temperature is in the vicinity of the absolute zero, it has been found that the current in the wire persists for a very great length of time. Obviously enough, the ordinary conception of resistance gathered in the temperature range of biological experience breaks down here.

The case is quite similar in the region of high temperatures also where chemical action is no more a mutual exchange of the outer electrons but assumes the character of nuclear transformation. The reason is pretty obvious; while at ordinary temperatures, the energy of chemical action is far too small to affect the nuclear stability at temperatures near a million degrees or so, the kinetic energy of the participants become comparable to that of the nuclear binding. Thus, we have in the latter case a veritable transmutation of the elements such as no alchemist might have dreamed of.

So, it would seem that the laws needed for a description of natural processes are by their very nature *restricted* to the range for which they hold good, becoming less and less true as we go farther away from that range. Considered in this light, commonsense represents only that body of knowledge which holds good in the range of sizes and dimensions comparable to our own, but breaking down for every other.

* Digest of an Address by Prof H. J. Bhabha, F. R. S., Director, Tata Institute of Fundamental Research, Bombay, at the Indian Institute of Science, Bangalore, on 14th Feb. 1950.