companied by a considerable spreading of the bands. However, some substances, e.g., streptomycin, which were immobile in the Partridge procedure, migrated rapidly and could be readily developed by the permanganate reagent. Further investigation of this approach would be profitable.

TABLE 1

R_f VALUES DETERMINED ON WHATMAN NO. 1 PAPER, USING BUTANOL LAYER AS THE ELUTING SOLVENT (DOWNWARD MIGRATION). TEMPERATURE ~ 25° C.

Name of compound	R_f value
α-Methyl glucoside	0.35
α-Glucose penta-acetate*	0.969
Mannitol	0.08
Sucrose	0.02
Melibiose	0.009
Lactose	0.013
Melezitose	0.01
Glucosamine	0.052
n-Carbobenzoxy-2-amino-α-methyl glucoside*	0.816
Methyl xylofuranoside	0.449
Salicin	0.526
Turanose	0.042
Potassium glucose-1-phosphate	0.003
D-Ribose	0.269

* Test substance was applied to the paper in ethanolic solution and dried before placing in the chamber.

In Table 1 are shown the R_f values of a series of compounds determined by using potassium permanganate.

References

1. BROWN, F. *et al.* *Nature,* Lond., 1948, **161**, 720.
2. KENT, P. W. and LAWSON, G. J. Paper in preparation.
3. PARTRIDGE, S. M. *Nature,* Lond., 1946, **158**, 270.
4. ——— *Biochem. J.,* 1948, **42**, 238.

Paper Chromatography of Proteins and Enzymes[1]

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The original work of Consden, Gordon, and Martin (*1*) on the paper chromatography of substances of biological importance has been extensively developed, and a large literature has accumulated which cannot be adequately summarized here. So far as we are aware, however, there is no report of the separation of protein molecules by paper chromatography.

We wish to report preliminary results of an investigation of the paper chromatography of proteins and enzymes. Our work shows that it is possible to study the movements of proteins on paper, and to determine the conditions under which proteins may be separated by this means. It is also possible by the same means to bring about separation of the components of enzyme mixtures, and to study enzyme activity, manometrically, on paper.

Our work has been greatly facilitated by the use of hemin as a "marker," the presence of a protein-hemin complex on paper being easily shown by the benzidine-hydrogen peroxide reagent. We employ buffer and aqueous salt solutions, instead of nonaqueous solvents, as developing agents.

The technique employed is that of Williams and Kirby (*2*), which involves chromatography by capillary ascent. Whatman No. 1 sheet filter paper has been used throughout this work. When drying is complete, the paper is streaked with freshly prepared benzidine reagent, using a small paint brush. Color development is immediate and intense, and should be photographed almost immediately, as a background color develops gradually and this tends to obscure the color from the protein-hemin complex.

Protein solutions. Ten mg crystalline protein is dissolved in 1 ml of distilled water or saline and to this is added 0.02 ml of 2.0% hemin, which has been dissolved in 3% sodium bicarbonate solution. Aliquots of 0.01–0.02 ml are used for chromatography.

Developing solutions. A search for suitable developing solutions has resulted in the exclusive use of aqueous solutions of salts and buffers. For buffers, 50 ml of a M/5 solution of the salt is adjusted to the desired pH with a *N*/5 solution of HCl or NaOH and the volume is made up to 200 ml. In the case of acetate-HCl buffers, normal solutions are used. Salt solution concentrations are usually in the region of 1–2%.

The use of organic solvents gave unsatisfactory results. *n*-Butanol saturated with water allowed extremely little movement of proteins and favored the formation of an immediate, deeply colored background on application of the benzidine reagent. Aqueous solutions of ethylene glycol, propylene glycol, ethanol, or acetone induced maximum or near maximum movement. All proteins tested moved about the same distance under these conditions.

Benzidine reagent. The preparation is made as follows: Equal volumes of saturated alcoholic benzidine solution and 3% hydrogen peroxide are mixed and made acid with glacial acetic acid. Fresh reagent must be prepared daily. Poor color development results if the hydrogen peroxide stock solution has decreased in strength.

Color development with protein-hemin complexes is generally blue, but may be purple, brown, or green. Some salts, e.g., magnesium sulfate and sodium phosphate, interfere with color development. Citrate, glucose, trisodium phosphate, and potassium hydrogen phthalate at some pH values give rise to a deep purple color.

Background color development varies considerably, depending on the ease with which the benzidine reagent is oxidized. Acid pH values tend to retard background color development, while alkaline pH values give rise to a deeply colored background. Photographs are made of the chromatograms at the optimal development of color. It is necessary to photograph the chromatogram while the paper is still damp.

Hemin. A stock solution of 2% hemin in 3% sodium bicarbonate solution was prepared and 0.02 ml was added to 1 ml of the protein solution. In the proteins studied,

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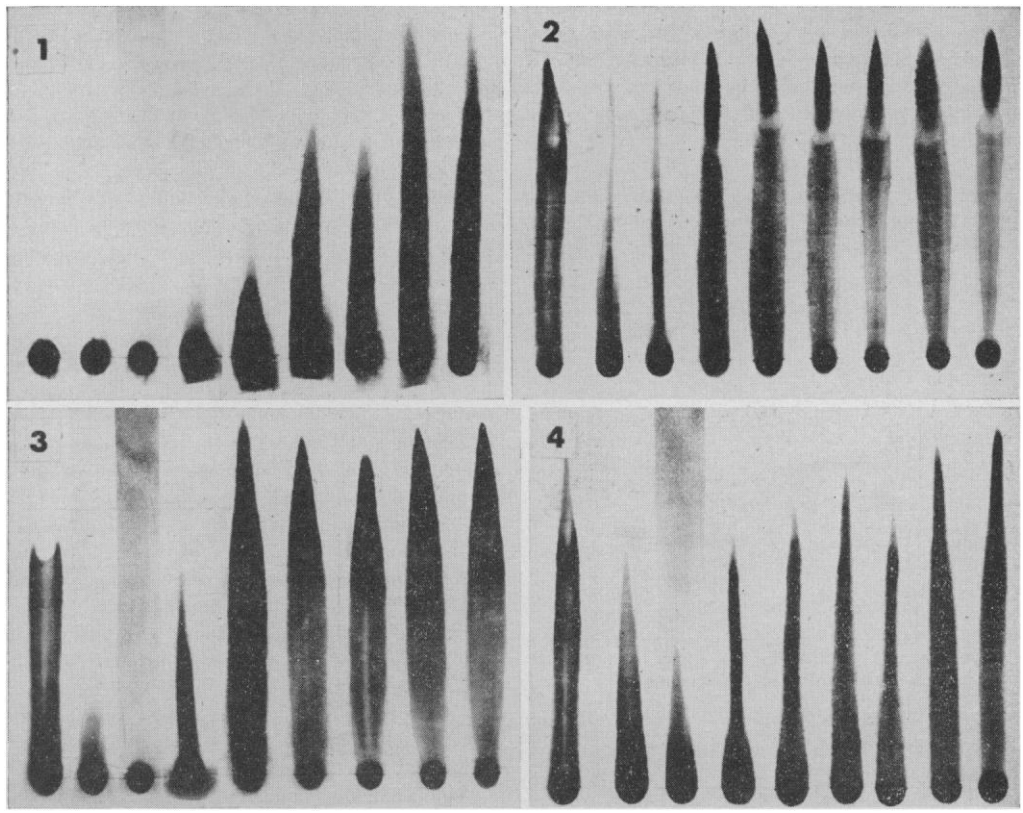
[2] Holder of a Canada Packers Research Fellowship of McGill University.



FIGS. 1–4. Composite photographs of unidimensional chromatograms of hemin and protein-hemin mixtures, showing the effects of changes of pH from 2 to 10 at 1-pH-unit intervals, from left to right. 1. Hemin control. 2. Bovine serum albumin-hemin. 3. β-Lactoglobulin-hemin. 4. γ-Globulin-hemin.

it has been observed that the presence of hemin does not alter the R_f value of the protein.

A control hemin chromatogram, in which 0.02 ml is diluted to 1 ml with distilled water, shows that movement is nil up to pH 5 and very slight at pH 6, and that it increases considerably above this pH (Fig. 1). Some developing solutions, among them those containing bicarbonate, glucose, sucrose, glycine, methionine, alanine, and urea, allow maximum or near maximum movement.

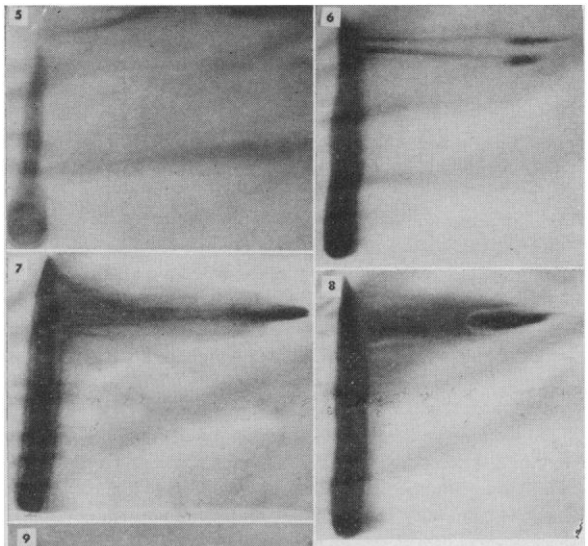
It has been found that some proteins and protein mixtures such as egg albumin, pepsin, papain, diastase, and urease will not combine with hemin. However, if hemin is added to a solution of these proteins, chromatographed, and streaked with the benzidine reagent, it is noticed that hemin remains at or near the point of origin (below pH 7). If the paper is allowed to stand for some time at room temperature, it is found that the background will color (to varying degree, depending on the nature of the salt in the developing solution), and a colorless spot will appear. It will be shown later that this colorless region actually contains the protein. Presumably the protein retards background color development and so produces a colorless "spot." Casein and β-lactoglobulin give rise to colorless spots or fractions up to about pH 5, and to deep blue fractions at higher pH values. γ-Globulin and bovine albumin give rise to colored fractions over the entire pH range (see Figs. 2, 4).

Time of development. It has not been found necessary to allow the solution front to advance beyond 20–25 cm, which requires about 90 min. The composition and shape of the chromatogram is determined within the first 20 min, and further development results in a more easily evaluated fraction. A complete two-dimensional chromatogram may be obtained within 5 hr.

The protein chromatogram. A long, rather narrow finger is formed, provided that the protein combines with hemin. The color intensity varies along with the finger, depending on the pH with which it is developed. Bovine albumin-hemin at pH 2 shows two intensely colored fractions inside the finger (Fig. 2). It may be shown that the greatest portion of the protein is concentrated in the leading fraction, and only traces remain in the finger.

One-dimensional chromatography results in the formation of a yellow-colored solution front, which fluoresces in ultraviolet light, and at pH 2, two separated bands appear at the leading edge of the chromatogram. Two-dimensional chromatograms result in a series of villus-like structures appearing in the second dimension. The presence of proteins on the paper tends to repress the full movement of those structures that are in the immediate vicinity of the protein.

Effect of pH. The variation of protein movement with change of pH seems to be much the same for all the proteins studied. At very acid pH values, e.g., pH 2, there



FIGS. 5–9. Two-dimensional chromatograms of boxine serum albumin-hemin under various buffer conditions: 5—pH 4.0, 6—pH 5.0, 7—pH 6.0, 8—pH 7.0, and 9—bovine serum albumin at pH 6.0 after the protein solution has been prepared for more than 36 hr, stored at 5° C, and then chromatographed with citrate in the first dimension and fumarate in the second dimension.

usually appears a well-defined leading section. In the region of pH 4–5 there is often little or no movement, and where movement does occur, no well-defined leading fraction occurs. Above this pH, a large, well-defined spot appears, which is quite constant in size. The R_f values depend upon pH in a similar way, being least around pH 4, which is near the isoelectric point of some of the proteins. R_f values and sizes of the spot are remarkably constant from pH 6 upwards.

Chromatography of bovine serum albumin-hemin complex. The R_f value of a bovine albumin-hemin complex is least in the region of pH 4. At pH 2, there appear two separated fractions inside the finger. This is also true at pH 3 but the spots are smaller and less intensely colored. From pH 5–12, there is a well-defined leading fraction, quite constant in size and position (Fig. 2).

In two-dimensional chromatograms, at pH 4, there is movement in the first dimension only (Fig. 5). At pH 5, with citrate-NaOH in the first dimension, and phthalate-NaOH in the second dimension, two separate fractions are obtained (Fig. 6). At pH 5.5 with citrate-NaOH in the first dimension and acetate-HCl in the second dimension, there appear two main fractions and a minor fraction. At pH 6 and 7 there is a single fraction appearing in the second dimension (Figs. 7 and 8).

It has been observed that if bovine serum albumin is allowed to remain in solution for a period of more than 36–48 hr, multiple fractions, sometimes as many as four, will appear. Various combinations of buffers have shown the separation of these fractions at pH 6 (Fig. 9).

It is apparent from these results that the crystalline bovine albumin which we have used behaves, when a solution is freshly prepared, as a homogeneous protein at pH 6–7, whereas two components are present at pH 5–5.5.

A private communication from Dr. G. Perlmann, to whom we are indebted for our supply of crystalline bovine serum albumin, states that this protein is homogeneous electrophoretically from pH 3.0 to 3.8 and pH 5.0 to 10.0. In the region of the isoelectric point the protein separates into two distinct electrophoretic components. This phenomenon seems to be a property of most serum albumins and indicates that crystalline albumin preparations contain several closely related proteins. Paper chromatography appears to confirm and may extend the results of electrophoretic analysis.

Chromatography of β-lactoglobulin-hemin complex. It is found that the union between β-lactoglobulin and hemin is not complete if the pH is 5 or less, i.e., the leading fraction is colorless. From pH 6 upwards, the complex shows as an intense blue. Thus the formation of a complex between this protein and hemin is dependent upon pH. R_f values show much less variation than those of bovine albumin. The R_f value is least at pH 4 and greatest at pH 6, where it is 0.86. All other values lie very close to 0.80 (Fig. 3).

Two-dimensional chromatograms between pH 6 and 7 demonstrate that this crystalline protein is not homogeneous. The use of citrate-NaOH in the first dimension and tartrate-NaOH, phthalate-NaOH, or acetate-HCl in the second, shows that there are a number of fractions in this sample. However, their complete separation under these conditions has not yet been achieved (Fig. 10).

We are again indebted for this sample of protein to Dr. Perlmann, who states that in 1945 this protein was found to be a mixture, there being two distinct electrophoretic components in the pH range 3.7–5.0, and also between pH 6.0 and 7.0.

Chromatography of human γ-globulin-hemin complex. It appears that γ-globulin, at the concentrations used, does not combine completely with hemin over the entire pH range. At each pH value, some combination takes place, but it is evident that from pH 3 to pH 8, there is a portion that does not combine, the amount of this portion decreasing with an increase in pH.

At pH 2, γ-globulin gives rise to two separate fractions inside the finger, in much the same manner as that shown by bovine albumin, but the fractions are smaller in size. Movement is least at pH 4. R_f values are 0.78 at pH 2.0 and 7.0, and increase to 0.81 at pH 10 (Fig. 4).

A two-dimensional analysis shows an excellent separation of two components of γ-globulin, the separation occurring at right angles. The first developing solution may be a phthalate-NaOH buffer at pH 6.0, 5% ammonium sulfate or 2% oxalic acid, followed by 2% tartaric acid in the second dimension (Fig. 11).

This protein, according to Dr. G. Perlmann, who kindly provided the sample, consists of one main electrophoretic component (96% of the total protein content), and it shows a marked boundary spreading, which indicates lack of homogeneity.

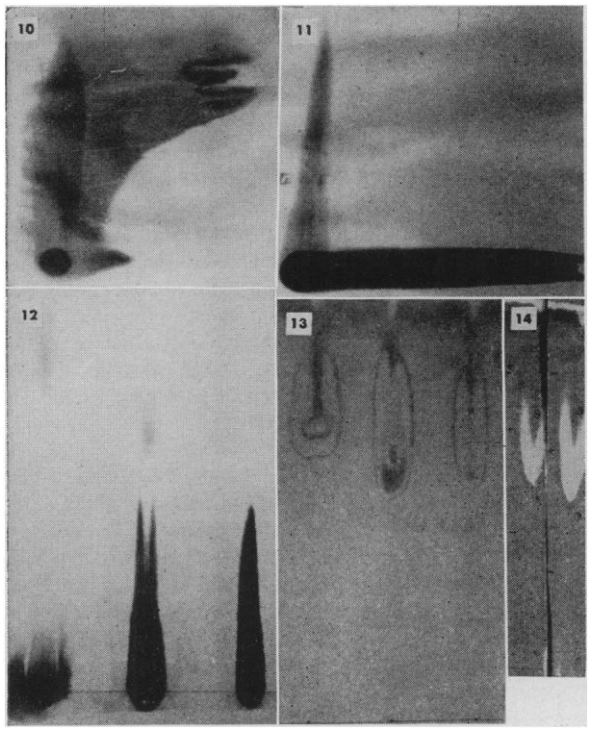


FIG. 10. Two-dimensional chromatograph of β-lactobulin-hemin at pH 6.5.

FIG. 11. Two- dimensional chromatogram of γ-globulin-hemin, using a phthalate buffer at pH 6.0 in the first dimension, followed by a 2% tartaric acid in the second dimension.

FIG. 12. Papain, papain-casein, and casein, in that order, developed with 2% succinic acid as the developing solution.

FIG. 13. Papain-bovine serum albumin, diastase-β-lactoglobulin, and β-lactoglobulin developed in a phthalate buffer at pH 5.0. There is no separation.

FIG. 14. Egg albumin developed in distilled water at pH 6.4. The resulting white spot was eluted, and identified by serological means.

Chromatography of enzyme preparations. Some preliminary work has been done on the movement of enzyme solutions. Preparations of pepsin, papain, malt diastase (commercial preparations from British Drug Houses, Ltd.) and urease (25-mg Urease-Dunning tablets) were used, and it was found that hemin will not combine with any of them. After streaking with the benzidine reagent and allowing the paper to stand for a time, it is observed that the malt diastase and papain preparations give rise to very well-defined, colorless oval spots. The urease preparation produces a vertical streak, but the pepsin preparation fails to show any definable movement. The papain preparation shows R_f values of 0.81 up to pH 3, which increase to 0.90 at pH 8.0–12.0. The malt diastase preparation shows a very constant R_f value over the entire pH range, varying only between 0.90 and 0.95, whereas the urease has an R_f value of 0.78 at pH 2, which increases to 0.85 at pH 3 and then gradually increases to 0.92 at pH 12. It seems likely that paper chromatography will be of service in the evaluation and examination of commercial enzyme preparations.

Chromatography of mixtures of proteins. One-dimensional chromatograms rarely separate a pair of proteins. We have succeeded, however, in separating components of a mixture of papain and casein, using 2% succinic acid as the developing solution (Fig. 12). The movement of papain is apparently retarded by the presence of casein. Other proteins have been run in combination with the preparations of papain and diastase, but no separations were effected (Fig. 13).

Chromatography of egg albumin. Crystalline egg albumin was used and, like the enzyme preparations already referred to, it will not unite with hemin. It appears as a colorless spot on a colored background (Fig. 14). It is barely detectable below pH 4, at which point it has an R_f of 0.60. This increases to 0.75 at pH 9.

The identity of the protein present in the colorless spot with egg albumin was shown by its ability to form a precipitin with a specific antiserum produced in a rabbit by albumin injections.[3] Precipitin formation was produced only by the solution of the substance contained in that part of the paper strip believed to contain the protein, as determined by the streaked control.

Our results prove that a solution of crystalline egg albumin may be chromatographed under the given experimental conditions, the position of the protein being indicated by a well-defined colorless region on a colored background. The protein present in this region reacts typically with an ovalbumin antiserum.

Chromatography of a urease preparation and the manometric estimation of urease on paper. The question arose as to whether enzymes will retain their catalytic activities if they are subjected to our chromatographic technique. A series of chromatograms was therefore set up for this purpose, the enzyme selected being urease.

The enzyme solution was prepared by grinding twenty 25-mg urease-Dunning tablets in a small mortar in 2 ml of a 2% cystein-glycine solution that had been adjusted to pH 6. It was then centrifuged for 15 min, and the supernatant solution was used for chromatography. Aliquots of 0.04 ml were applied to the paper by placing 0.02 ml on the paper, allowing it to dry, and adding another 0.02 ml. The developing solution was prepared by dissolving 2 g cystein hydrochloride in water and adjusting the pH to 6 with caustic soda solution, making the volume to 100 ml, and then adding 1 g glycine. This reagent should be prepared just prior to use. The solution was allowed to advance about 20–22 cm. The paper was then removed and allowed to stand at room temperature for a few min. While the paper was still damp, it was cut into 3-cm sections, starting 0.5 cm from the bottom of the paper. The boundaries were marked 7 mm on each side of the point of application of the aliquot.

Each section of filter paper was then cut into four parts, all of which were placed immediately in a Warburg manometric vessel with 3 ml acetate buffer at pH 5, and 0.2 ml 10% urea was placed in the side arm. As a control, a ten times dilution of the original supernatant was

[3] This antiserum was kindly prepared for us by Prof. E. G. D. Murray, of the Department of Bacteriology, McGill University.

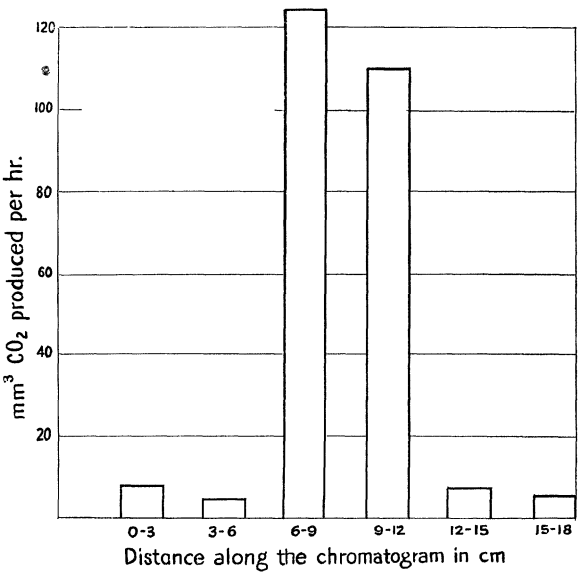


FIG. 15. Urease activity along the chromatogram.

used (diluted with physiological saline) and 0.4 ml of it placed in the Warburg vessel with 2.6 ml buffer and 0.2 ml 10% urea in the side arm. A period of 30 min was allowed for the contents of the vessel to attain thermal equilibrium (37° C). The urea in the side tube was then tipped into the vessels, and carbon dioxide output rate measured manometrically in the usual way for 1 hr.

The results show the existence of a distribution curve of urease on the paper strip. Traces of enzyme are left at the point of origin, but a fair proportion ascends the paper, whose maximum urease activity lies between 6 and 12 cm from the origin. On either side of the maximum there is a drop in enzymic activity. These results are shown in Fig. 15. If a parallel chromatogram is streaked with the freshly prepared benzidine reagent, it is found that the greatest extent of the colorless region on the colored background is 6–12 cm from the origin.

On assessing the activity of the urease over the entire strip, by adding together the activities of the various parts of the strip it is found that the total activity amounted to 85% of that expected from the amount of urease placed on the paper. Another assessment of the total urease activity over the entire strip showed a recovery of 110%, the assessment being made from a calibration curve previously prepared, relating activity (rates of CO_2 production) to the quantity of urease. These results show that urease activity is not diminished within experimental error by our chromatographic technique, and that movement of the urease molecule on filter paper can be followed. It is evident, however, from our preliminary results that metallic constituents of the filter paper may appreciably affect the rate of movement of urease and possibly other proteins. This needs more study.

Chromatography of human serum. A few experiments have been carried out on the chromatography of human serum. The results show the presence, at pH 6, of a complex mixture of hemin-reacting proteins. Three of the fractions appear to give R_f values identical with those found with a preparation of crystalline human serum albumin. Human serum globulins do not seem to move appreciably in the second dimension. We estimate that, with our technique, between 6 and 10 protein fractions appear. This work is now being extended with a view to discovering whether paper chromatography of blood serum may be used for diagnostic purposes.

References

1. CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P. *Biochem. J.*, 1944, **38**, 224.
2. WILLIAMS, R. J. and KIRBY, H. *Science*, 1948, **107**, 481.

Tetrazolium Chloride as a Test for Damage in Artificially Cured Peanuts

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The artificial curing of peanuts is being investigated in several Southeastern states as one phase of a mechanization program designed to reduce production costs and improve nut quality. Drying methods found to be practicable for certain other oil crops and for cereal crops could not be applied empirically to the peanut because of the uncertainty of curing effects upon flavor of nuts for the food trade and because of the heat- and moisture-insulating properties of the peanut shell. Large scale experiments on artificial curing of peanuts, therefore, have had to await results of preliminary trials.

In a series of exploratory curing tests at the Alabama Agricultural Experiment Station, using different drying temperatures and rates of air flow, the desirability of a quick test of curing effects upon peanut quality soon became apparent. It was evident that the standard tests of quality in sound, mature peanut kernels—free fatty acid percentage, iodine value, and germinability—all required more time and analytical apparatus than were practical for rapid estimates of heating effects upon peanuts. Furthermore, germinability could be used at time of harvest only for the Spanish variety of peanut. The runner variety, which constitutes the great bulk of the Alabama crop, germinates only after a period of dormancy of several weeks following harvest.

However, it was assumed that seed viability might be correlated with some of those properties of flavor and oil quality which characterize peanuts of high market value both at time of harvest and after storage. Successful use of tetrazolium salts as viability stains in seed germination experiments (*2, 3, 5, 7*), in determination of vitality in miscellaneous plant and animal cells and tissues (*6*), and in demonstration of injury by heat in stem tissues (*8*) and by freezing in maize (*1, 4*) suggested their possible application as a quick test for heat and drying damage in artificially cured peanuts. Preliminary results indicate that they may be so used.

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