THE DETECTION OF JAFFÉ-POSITIVE SUBSTANCE IN BLOOD SERUM BY PAPER PARTITION CHROMATOGRAPHY

THE PROBLEM OF APPARENT CREATININE

by

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The colour reaction between creatinine, picric acid and alkali, also called the *Jaffé reaction*[1], after its discoverer, was the basis for the elaboration of the quantitative colorimetric method of O. FOLIN[2, 3, 4, 5, 6]. This reaction is not specific, since it is influenced by several substances present in the reaction mixture (*e.g.*, in urine and serum).

Many of these substances are themselves Jaffé-positive: glycocyamidine[7, 8, 9, 10, 11], hydantoin[9, 10, 12, 11], methylhydantoin[12, 10, 12], acetone[1, 14, 8, 9, 10, 12], acetoacetic acid[15, 9, 10, 16], pyruvic acid[17, 16], laevulic acid[10, 17], acetylacetone[17], and ethyl acetoacetate[9, 17]. Italian authors[18] discuss Jaffé-positive substances in connection with adrenaline, glutathione, tryptophan and histidine; other authors were not at all able to show the Jaffé-positive behaviour of tryptophan[19, 20, 17] and histidine[19, 21] and photometric estimations carried out with histidine[17] show rather that it is practically Jaffé-negative. Jaffé-positive behaviour, especially *after heating*, is usually mentioned in the cases of glucose[1, 9, 10], fructose[9, 10], lactose[9, 10], galactose[10], arabinose[10], Dopa[10], glucosamine[9, 19, 10], the lactone of homogentisic acid[10], glycylglycine anhydride[9, 19*] and uric acid[1, 10]. According to other authors[12, 17, 14] Jaffé-positive behaviour is shown by the majority of the substances mentioned in the *cold* only after some time. Other substances only influence the *kinetics* of the reaction[22, 20, 23, 24, 25]: histidine, glucose, adrenaline, ascorbic acid, guanidine carbonate, dimethylguanidine hydrochloride, and diketopiperazine. Furthermore a larger number of various compounds[19, 9, 12, 20, 17] was examined with respect to Jaffé-positive behaviour, with positive and negative results which it is not necessary to discuss since their occurence in blood serum is improbable. Among others, the following compounds were shown to be Jaffé-negative: glycine, alanine, leucine, tyrosine, cystine, cysteine, proline, asparagine, glutamine, urea, arginine, glycine ester, etc.

Under these circumstances the analytical values for creatinine in biological material actually represent *apparent creatinine* only (apparent creatinine = creatinine + non-creatinine component).

Some modifications of the original method attempted to eliminate the influence of the chromogen (non-creatinine component) by specific enzymic destruction of creatinine using *Corynebacterium ureafaciens*[26, 27] as well as, to some extent only, by absorption of creatinine on LLOYD's reagent (followed by elution according to O. H. GAEBLER[28] or H. BORSOOK[29]) or by its conversion into the Jaffé-negative bis-3.N[2]-hydroxymethyl-creatinine[30, 31].

* W. WEISSE AND C. TROPP[12] noticed Jaffé-positive behaviour in the cold. J. SCHORMÜLLER AND H. MOHR[17] carried out quantitative estimations photometrically and their calculations gave quantities that were so small as to cause them to consider glycylglycine anhydride to be practically Jaffé-negative. This was also shown by A. F. RICHTER[32].

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The attempt to elaborate an exact *chemical* method and to ascertain all interrelations in the complicated medium of the biological material has previously caused both E. ABDERHALDEN AND S. BUADZE[22], and K. DIRR AND H. L. SCHADE[20], to ascertain the influence of various substances on the course of the reaction. According to these authors histidine[22] and guanidine compounds[20] inhibit the reaction (histidine lowers the amount of creatinine found up to 20%), whereas glycylglycine anhydride[20] increases this amount; tryptophan and aspartic acid[20] do not interfere. This problem was studied more systematically by A. F. RICHTER and coworkers[21–35, 21, 23–35], who, especially by accurate study of the kinetics of the Jaffé-reaction, showed an activating effect of adrenaline and ascorbic acid and an inhibiting effect of histidine and glucose. Experiments carried out with beef serum showed furthermore that 20% of the apparent creatinine is accounted for by the Jaffé-positive, non-creatinine component which, according to the authors, cannot be accounted for by any of the known Jaffé-positive substances occurring primarily in physiological media.

One can therefore sum up the *present state* of the problem as follows: the colour reaction of creatinine is influenced

1. indirectly by certain substances showing either small Jaffé-positive behaviour or none at all, but, whatever the case may be, causing activation or inhibition;

2. by Jaffé-positive substances (one or more), which would suffice to explain directly and quantitatively its colour intensity and the amount of the non-creatinine component. The existence of such a compound has not yet been doubted.

Since up to now all experiments were mostly directed, with more or less success, towards the effect of the mutual interaction of compounds accompanying creatinine, we decided to study the second possibility, *i.e.*

a) to make sure whether blood serum does contain, in addition to creatinine, a substance exhibiting any marked Jaffé-positive behaviour;

b) to identify this substance.

The application of the method of paper chromatography as elaborated by R. CONSDEN, A. H. GORDON, AND A. J. P. MARTIN[36] appeared to be ideal for this problem. The paper chromatography of creatinine was described, or at least referred to by C. E. DENT[37], by R. G. WESTALL[38], and more extensively by G. A. MAW[39, 16].

Using collidine or sec. butanol G. A. MAW could detect creatinine and creatine (after its conversion to creatinine) in quantities down to 1 μg. He confined his biological material to urine and muscle extracts. Under the interfering substances he found glycocyamidine, pyruvic and acetoacetic acid, all Jaffé-positive, but having very different rates of colour formation with sodium picrate. The detection of these substances has not been attempted in biological material.

The creatinine content of blood is roughly hundred times less than that of urine. Hence it follows that the direct use of serum is, in our case, impossible; even if we neglect the possibly troublesome interference of proteins that have not been removed, it would be necessary to place a large amount of material (100–500 μl, which by itself would be inconvenient) on the paper to detect creatinine at all in such small quantities by a colour reaction. Therefore we concentrated the serum after deproteinization to $^1/_{10}$ of its original volume and this solution was placed on the paper. The amount of creatinine treated in this manner (7.5 μg to 15 μg in 75 μl — or actually less) gave us some certainty that even different Jaffé-positive substances, if these could be expected to be present, would show themselves under such circumstances.

A. PAPER CHROMATOGRAPHY OF BLOOD SERUM

1. DEPROTEINIZATION

Deproteinization of serum is a problem of fundamental importance[18, 31]. From a number of methods, *ultrafiltration* was selected as a relatively ideal and chemically

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mildest method, although even here the loss of certain substances by stronger adsorption in the pores of the ultrafilter cannot be entirely excluded.

a. Deproteinization of serum by ultrafiltration

For ultrafiltration membrane filters were used which were prepared by immersing the filter paper in a collodion solution and their permeability was tested by means of dye-stuff solutions[40] (congo-red, neutral-red, methylene-blue).

The best result was obtained with an ultrafilter prepared by double immersion into 4% collodion; after repeated washing not only proteins but also practically all peptides were retained, whereas under the same conditions the ultrafilter once immersed already allows a small part of the peptides to pass. The use of 2% collodion was found to be unsuitable.

The preparation of serum for chromatography

10 ml of serum diluted to the threefold volume were ultrafiltered in the Bechhold apparatus under a pressure of 3 to 5 atm N_2 (velocity 5 to 10 ml of liquid per hour). For more perfect washing of crystalloids three portions of water (each portion = approximately 30 ml) were added until the final volume of the ultrafiltrate had become 100 to 110 ml. The qualitative test for the presence of proteins in the filtrate by means of trichloroacetic acid was negative, but strongly positive in the residue on the filter.

The ultrafiltrate was then evaporated to dryness in a vacuum (40° to 50° C) and to the yellowish residue 1 ml of distilled water was added; the mixture was then quantitatively transferred as far as possible into a microtest-tube and centrifuged. The clear supernatant (p_H 8–9) over the insoluble residue was decanted and used for chromatography*.

Although ultrafiltration by means of membrane filters was the most convenient method of those tried by us (dialysis, electrodialysis, ultrafiltration by means of a BERKEFELD filter covered with collodion) a further attempt was made to replace it by deproteinization by chemical reagents and in this manner to eliminate, at the same time, the possibility of possible changes in the solution during this somewhat tedious operation. From a number of deproteinizing agents *acetone* was selected, chiefly for its quick and perfect deproteinization ability. The criterium of its suitability was the comparison of the chromatogram of the "acetone filtrate" (Ac-filtrate) with the parallel chromatogram of the "ultrafiltrate" using the latter as standard.

b. Deproteinization of serum with acetone

10 ml of serum were slowly precipitated with 100 ml of redistilled acetone (reducing substances were removed by boiling it with $KMnO_4$); the protein precipitate was centrifuged and vigorously shaken with a further 50 ml of acetone. This operation was repeated once more.

The Ac-filtrate *is worked up* in a manner analogous to that used in the case of the ultrafiltrate. After centrifuging the insoluble, markedly yellow residue forms an upper layer in the liquid which has the usual colloidal appearance with a faint milky opalescence; the p_H of dissolved fraction is about 7.

2. CHROMATOGRAPHY OF THE SOLUBLE FRACTIONS OF THE ACETONE-FILTRATE AND THE ULTRAFILTRATE

a. Chromatography from a mixture of butanol-acetic acid-water

Reagents: n-butanol (b.p.117° C);
glacial acetic acid;
picric acid recryst. according to S. R. BENEDICT[41]; 7.5 ml of sat.solution diluted with water to 10 ml;
3% NaOH.

* The insoluble residue, chiefly that which was obtained in the course of the acetone deproteinization procedure, has, after a few preliminary attempts at chromatography on paper strips, been left open for further investigation. Although it was not always possible to obtain consistent results, the presence of one or two Jaffé-positive substances (one of which may be creatinine) appears to be probable.

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For mutual comparison of the chromatograms a volume of 30 ml of serum was taken, which, after dividing it into three parts, was deproteinized as follows:-

1a. by ultrafiltration with collodium filter (4%) *twice* immersed;
1b. by ultrafiltration with collodium filter (4%) *once* immersed;
2. by *acetone*.

For one-dimensional chromatography Whatman No. 1 filter strips of the approximate dimensions 2 cm × 30 cm were used. Of each fraction 25 μl, 50 μl and 75 μl were placed on the filter paper and to shorten the time of drying the evaporation of water was accelerated by blowing a strong current of air from a ventilator underneath the strips. For chromatography the samples were pipetted within 2 hours and the filter paper strips placed in a glass cylinder (C. E. DENT[42]). As a medium from which the chromatograms were run we used the mixture n-butanol-acetic acid-water (BuAc-mixture) in the ratio 40: 10 : 50 respectively, according to S. M. PARTRIDGE[43, 44], the upper layer of which, obtained after shaking and separation, was used in the trough.

After 10 to 12 hours the chromatography was interrupted and the paper strips were developed after 10 minutes drying in air by fine spraying with alkaline picrate: a freshly prepared mixture of 1 part 3% NaOH + 1 part picric acid solution (the concentrations of both components were chosen in accordance with the work of R. W. BONSNES AND H. H. TAUSSKY[45] on the quantitative estimation of creatinine, although all experiments carried out with a preparation of synthetic creatinine with gradually varying concentrations of picric acid and alkali gave approximately equal results; for application to biological material we naturally always tried to avoid the use of strong alkali).

The chromatograms of all three samples obtained by various deproteinization methods showed an absolutely identical location of the spots (Fig. 1a).

An informative experiment on the removal of salts by means of exchange resins (Wofatit K and M) did not give ideal results and Zeocarb. 215 and Deacidite as used by S. M. PARTRIDGE[43] were unfortunately unobtainable. Nevertheless, in the majority of sera all the inorganic salts settled at approximately $^1/_7$ of the entire length of the chromatogram and never exceeded $^1/_6$ of this distance, if perfectly purified, redistilled water was used for dissolving the residue after evaporation (Chemically Pure Water, Abbot Lab.)*.

The identity of the Ac-filtrates and ultrafiltrates, demonstrated in the case of several samples of blood serum, finally decided us to use acetone deproteinization only; the entire process is shortened by this procedure (up to 2 days) and furthermore the neutral reaction of the soluble fraction prevents such possibilities of changes as might occur in the alkaline medium of the dissolved fraction of the ultrafiltrate.

The extraction of the dry matter by half the amount mentioned above or, on the contrary, by three times more water did not show any difference in the chromatographic pattern from that of Fig. 1a.

The extent to which the concentration of acetic acid has any effect on the separation of the substances was examined by using the three-component system butanol-acetic acid-water in the ratios: a. 40 : 10 : 50
b. 20 : 10 : 50
(in the case of the ratio 20 : 20 : — no separation was obtained even after addition of a large amount of water). This effect makes the appearance of the chromatogram, obtained with n-butanol alone, still more pronounced.

b. Chromatography from butanol, amylalcohol, and its isomers in all samples of sera show only the presence of one main spot of larger size ($R_{F\ (15°\ C)}$ = 0.13). The salts do not move at all and form a circle around the spot where the sample has been placed. In the case of higher concentrations (50 to 100 μl) the main spot slowly merges with the area of the inorganic salts (similar concentrations chromatographed from BuAc-mixture under similar circumstances immediately showed two distinctly separated spots). The chromatography from these solvents is therefore rather problematic for the solution of the given problem, since the resolution of the individual components, if occurring at all, is evidently poor.

c. Chromatography from other solvents did not give any better results than those obtained with the BuAc-mixture.

In the case of *chloroform* spot II, just above the solvent front, was only visible in one of three cases and was rather indistinct; more interesting is the intense orange colouring of spot III in the solvent front, which itself was somewhat brownish. In the case of the remaining two samples, the colouring of these parts was not at all decisive. The chloroform front on the blank strip was not

* After the conclusion of our study, our attention was drawn by C. E. DENT to the desalting apparatus.

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coloured at all. The orange-red spot I round the starting point indicates the presence of a substance which has no movement in this solvent (*e.g.*, creatinine).

Spot I, appearing in *benzyl alcohol* runs only when higher concentrations of serum are used, merges with the main spot II (very probably creatinine) and appears to originate from a different substance.

s-Collidine (mixture with *2.4-lutidine*), *m-cresol*, *phenol*, *methylenechloride*, a *mixture of pyridine and amyl alcohol* (with water 1 : 1 : 1 and without water), *butanol-ethanol* (1 : 1 : 1), *decalin*, *bromoform*, *benzene*, *toluene*, *xylene*, *petroleum ether*, and *ethyl acetate** were not very suitable for the separation of Jaffé-positive substances in blood serum.

d. Conclusion

In experiments that led to the identification of substances, chromatography from BuAc-mixture was used throughout. The appearance of the chromatogram with a sample of 75 μl is shown in Fig. 1a. The *spots I* (*unknown component*, $R_{F\ (15°\ C)} = 0.20$) and *II* (*supposed creatinine*, $R_{F\ (15°\ C)} = 0.38$) become coloured immediately after

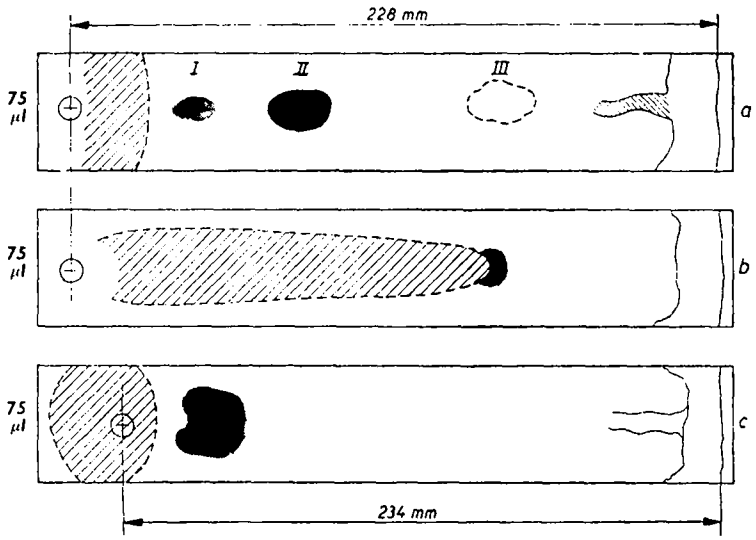

Fig. 1. Chromatogram of Ac-filtrate of serum:
a. from BuAc-mixture (40 : 10 : 50)
b. from BuAc-mixture (20 : 10 : 50)
c. from n-butanol.

development with alkaline picrate. The unknown component is located at about one-half of the creatinine distance from the place of administration of the sample: when the fronts of inorganic salts reached this position, as happened in earlier experiments, spot I moved along with it and was found close to this front. *Spot III* becomes coloured after 3 to 6 hours, when it is very faintly visible (seen better against the light) and is therefore *unimportant* for the solution of our problem.—The *dark band* close the front of the solution solvent corresponds to *cholesterol*, as was shown by means both of the SALKOWSKI and LIEBERMANN-BURCHHARDT reactions carried out directly on the chromatogram.

Here it is important to remember that *volatile Jaffé-positive compounds*, which may be present in serum, are removed during evaporation of the Ac-filtrate as well as during the placing of the sample on the filter paper.

* All the solvents were saturated with water

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B. EXAMINATION OF THE JAFFÉ-POSITIVE COMPONENTS

1. THE IDENTIFICATION OF CREATININE

The identity of spot II with creatinine was confirmed by two methods:-

a. by reaction with *3.5-dinitrobenzoic acid* (3.5-DNB);

b. by chromatography with an *addition of synthetic creatinine.*

The reaction with *3.5-DNB*, recommended mainly for its great sensitivity, was carried out on the chromatogram with the soluble fraction of Ac-filtrate (75 μl) by spraying with the reagent mentioned (recrystallized according to A. P. JANSEN, W. SOMBROEK, and E. C. NOYONS[46]) in a concentration of 0.3 g/5 ml ethanol + 5 ml of 10% NaOH; it is important always to have the solution freshly prepared, on standing for some time a precipitate of the Na-salt begins to form (addition of alcohol somewhat increases its solubility). The place of localized creatinine is shown by a distinct blue-violet colouring of spot II which attains a maximum within a few seconds and then slowly fades. The reaction is sensitive and permits detection of 0.5 μg to 1 μg of creatinine.

The *Weyl reaction*, based on the formation of a red to reddish-violet colour by creatinine and sodium nitroprusside in alkaline medium, was found to be most suitable with an 1% aqueous solution of sodium nitroprusside with which a spot of synthetic creatinine was sprayed. After drying the colour was developed with 5% NaOH; 5 μg creatinine could be detected by a distinct orange-pink colour, 1 μg was very faintly visible and the colour disappeared after a few seconds.

However, the application of WEYL's reaction to the detection of creatinine on the chromatogram of sera was a complete failure, probably because of the effect of traces of acetic acid in the fibres of the filter paper (BuAc-mixture!) which causes the colour to turn from orange-red into yellow.

The spot of synthetic creatinine *added* in concentrations of 2.5 μg and 5 μg to the soluble fraction of the Ac-filtrate placed on the filter paper (40 μl and 75 μl) exactly coincided with spot II.

2. THE UNKNOWN COMPOUND

a. Examination of synthetic products for Jaffé-positive behaviour

The Jaffé-positive behaviour of compounds was frequently investigated by various authors, usually under different conditions (temperature, sodium carbonate instead of alkali, etc.). Since the results obtained depend entirely on these factors it was necessary to check several data under such conditions as were used in the development of the chromatograms.

From the possible substances we mainly selected blood components with the exception of those that only form part of the erythrocytes and are not contained in the plasma (serum): glutathione, ergothioneine, pyrocatechol, hydroquinone, bile acids, oxalic acid, formic acid, trimethylamine, guanidine, methylguanidine, acetone, acetoacetic acid, and β-hydroxybutyric acid. Because of their very small concentration in sera adrenaline, indicane, acetylcholine, bilirubine, and urobiline were excluded. Citrulline and rhamnose were not available. Fatty acids and phospholipids were not considered in these experiments. On the other hand, glycocyamidine, hydantoin and methylhydantoin, although their existence in the serum has not yet been ascertained, were also tried.

All three substances were prepared synthetically. Glycocyamidine and methylhydantoin were only obtainable in very small amounts so that the amounts placed on the filter paper were very slight. All three compounds are Jaffé-positive, their colour increases with the time and attains an intensive orange-red shade within a few hours.

Spot reactions. The compounds were placed on the paper (Whatman No. 1) and the colour reaction was developed by spraying with alkaline picrate of the usual concentration.

The compounds used, as well as a review of the results, are recorded in Table I.

Paper chromatography. From the substances included in Table I some cannot be the unknown component found in the Ac-filtrates as they are either Jaffé-negative or

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TABLE I

COMPARISON BETWEEN THE AMOUNTS REPORTED FOR SERUM OR BLOOD AND THE JAFFÉ-REACTION ON PAPER

Substance	Concentration in human blood serum mg%	Concentration in the soluble fraction		Sensitivity of colour reaction			
		γ/50 µl	γ/100 µl	Amount γ	Immediat.	After 15′	After 30′
Glycine (MERCK-SÖRENSEN)	5–8–10 N (tot.)			25 50 100	Negative		
α-Alanine (MERCK)					Negative		
Valine (MERCK)					Negative		
Leucine (MERCK)					Negative		
Histidine-HCl (HOFFMANN-LA ROCHE)	1–2	5–10	10–20	5 10 20	Ø Ø Ø	Ø (+) (+)	Ø (+) (+)
L-tyrosine (MERCK)	1–2	5–10	10–20	5 10 20	Ø Ø Ø	Ø Ø Ø	Ø Ø Ø
Histamine–2HCl (HOFFMANN-LA ROCHE)	0.025–0.5	0.125–2.5	0.25–5	0.25 5	Ø (+)	Ø Ø	Ø Ø
Glutamine (SCHERING-KAHLBAUM)	6–10	30–50	60–100	50 100	Negative		
* Glucose (HEISLER)	60–120	300–800	600–1200	300 600 1200	(+)	+ to ++	++ to +++
* Fructose (SANDOZ-puriss.)	1–2 approx.	5–10	10–20	10 20	Ø Ø	Ø (+)	(+) (+) to +

* d-glueosamine-HCl (FRAENKEL-LANDAU)	75–115	370–600	750–1200	400 700 1200	Ø Ø Ø	Ø Ø (+)	Ø (+) (+) to +
l-ascorbic acid	0.6–2.4	3–12	6–24	3 24	Ø Ø	Ø +	Ø +
Pyruvic acid (MERCK)	0.5–1.2	2.5–6	5–12	2 6 12	Ø (+) +	+ to ++ + to ++ + to ++	After 3 hours + to ++ +++ +++ to int.
Uric acid (MERCK)	0.3–4	1.5–20	3–40	20 40	Undistinct		
Lactic acid (U.S.P.)	5–20	25–100	50–200	50 100 200	Negative		
Citric acid (MERCK)	2.4	12	24	12 24	Negative		
Urea (SPOLEK CHEM. a HUTNÍ)	15–40	75–200	150–400	200 400	Negative		
Glycogene pure (SCHERING-KAHLBAUM)	3	15	30	15 30	Negative		
Cholesterol (MERCK)	30–120	150–600	300–1200	150 300 600	Negative		
Cholin-chloride (SCHERING-KAHLBAUM)	0.2–2	1–10	2–20	10 20	Negative		

* During several hours the colouration increases and then turns to brown.
The scale of colour intensity: (+) nearly indistinct, + very weakly orange, ++ weakly orange, +++ orange, int. intensive.
Negative = Ø

too faintly positive to be seen in amounts taken for paper chromatography (histamine, tyrosine, glucosamine, fructose, histidine).

Only those compounds were used for paper chromatography which in the given concentration range had distinct Jaffé-positive behaviour without regard as to whether their colour appeared immediately or after some time. It was our task to find their movement (R_F values) from BuAc-mixture and therefore to exclude from further examination those compounds the location of which in the filter paper, using the given solvent, did not coincide with that of the unknown compound.

Uric acid (20 μg and 40 μg) — indistinct chromatogram from BuAc-mixture. Only after several days a very faint colour was observed; at about $^1/_1$ there was a pale spot (inorg. component).

Ascorbic acid from BuAc-mixture, even in the maximal concentration of 24 μg, was hardly visible at all. $R_{F\,(18.5^\circ C)}$ — 0.41, close to creatinine.

Glucose in a concentration of 600 μg separated from BuAc-mixture in the highest part of the chromatogram. A very slow colour was observed, only appearing after 30 minutes.

Pyruvic acid is the most interesting compound observed in the study of this problem.

In chromatography from BuAc-mixture the synthetic pyruvic acid "Merck" (in an amount of 12 μg) divided into 4 spots situated at nearly equidistant points. A short time after development spot III appeared and the very weak spot I; after 6 hours the following colouring was observed: I. very weak to weak, II. very weak, III. weak, IV. weak. After several days spot I and III had nearly disappeared and only spots II (now somewhat more intensively coloured) and IV stayed permanently.

Likewise even sodium pyruvate "Merck-for scientific purposes" could not be shown to be a perfectly homogeneous substance; at higher concentrations (60 μg) spot III slowly merges with spot I.

R_F values of individual spots measured at 15° C:-

	free acid	Na-salt
I	0.14	0.13
II	0.36	—
III	0.51	0.51
IV	0.69	—

Chromatography of the free acid from n-butanol itself shows the presence of two unseparated spots.

Hydantoin, methylhydantoin, and creatinine separated very well from n-butanol (a) and to a lesser extent from BuAc-mixture (b):

	a	b
Creatinine ($R_{F\,(15^\circ C)}$)	0.15	0.44
Hydantoin ,,	0.28	0.46
Methylhydantoin ,,	0.46	0.61

Glycocyamidine from BuAc-mixture merges with the creatinine spot; $R_{F\,(15^\circ C)}$ = 0.43. (See also [16]).

Conclusion

None of the last-named substances showed any identity with the unknown component judged by its location on the filter paper (Fig. 2).

As the Jaffé-negative substance *creatine* approaches the R_F of the unknown serum component more closely than any other substance considered up till now, and the possibility of partial anhydrization of creatine during the procedure (by acetic acid during chromatography, during drying-

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etc.), is mentioned by G. A. MAW[89], parallel chromatograms on one strip of Whatman No. 1 paper were run in the following manner:-

a. 30 μg creatine,

b. 30 μg creatine + 5 μg creatinine from BuAc-mixture. After slowly drying in air (10 to 15 minutes), the part containing a. was cut off and placed in a drying chamber at 100°-115° C for about 10 minutes and afterwards developed with alkaline picrate: intensively orange-red spot (creatine → creatinine) of $R_{F\,(15°\,C)} = 0.36$; part b. was developed with alkaline picrate in a normal manner and showed only a spot of creatinine of $R_{F(\,15°\,C)} = 0.45$.

If we compare the location of *hydantoin* and *methyl-hydantoin* on the chromatogram from butanol with the appearance of the chromatogram of the Ac-filtrate from the same solvent, we believe we can confirm other observations that these compounds are practically absent from serum, or at least not detectable by the Jaffé reaction.

The presence of *glycocyamidine* could not be either confirmed or disproved with certainty; but later experiments with glycocyamidine added to the soluble fraction of the Ac-filtrate before chromatography seem to provide evidence against its existence in serum with the assumption, of course, already made in the case of hydantoin and methylhydantoin.

The sensitivity of the Jaffé-reaction for *ascorbic acid* is too low to enable its detection in serum by this manner, even in maximal concentrations.

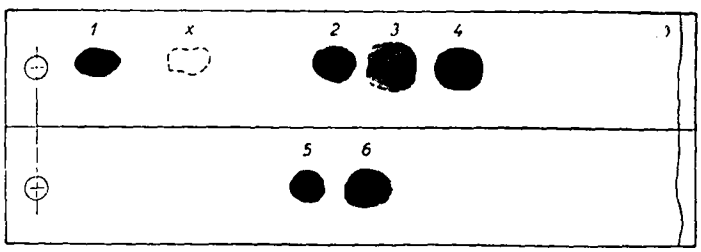

Fig. 2. "Model chromatogram" of synth.products from BuAc-mixture
1. Glucose
2. Creatinine
3. Na-pyruvate
4. Methylhydantoin
5. Glycocyamidine
6. Hydantoin
x. Approximate localization of the unknown component of serum

The presence of four spots in the chromatogram of *pyruvic acid* or its *sodium salt* seems to be due rather to the lack of purity of the preparation, although its source (*e.g.*, "Merck-for scientific purposes") does not favour this explanation.

Any explanation ascribed to the action of acetic acid contained in the solvent mixture on pyruvic acid, causing its polymerization, etc. seems to be ruled out by the occurrence of at least two spots, even when butanol without acetic acid is used.

In comparing the positions and appearance of the spots given by chromatography of the acid and its salt, pyruvic acid most probably seems to be located in the spot III in both cases. This has been confirmed by subsequent polarographic estimation.

Our observations are interesting considering the observation of G. A. MAW who does not mention any multiplicity of spots in the chromatography of pyruvate. This could be explained by the superior purity of his preparation. Sec. butanol, as used by G. A. MAW, was not available to us. No less interesting is his remark that pyruvic acid did not give bands when present in amounts much less than 100 μg. We were able to detect spot III when chromatographing only 6 μg of the acid or of its salt. The appearance of several spots requires some further elucidation* although these experimental results of this *partial* problem were of no fundamental importance for the solution of our ***main*** problem.

b. Identification of unknown compound

Since the unknown compound did not seem to be either an aminoacid or a sugar (negative ninhydrine and $AgNO_3$-reduction tests on the place of the localized spot on

* The case might be analogous to that of aspartic and glutamic acid, where further research has been suggested by C. E. DENT.

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the serum chromatogram), we decided to reinvestigate pyruvic acid (sodium salt) owing to its problematic behaviour during chromatography.

A chromatogram from BuAc-mixture with a mixture of 5 μg creatinine + 12 μg sodium pyruvate was started, and then pyruvic acid located itself *under* the creatinine spot.

An unsolved problem which led to the identification of the unknown component, however, remains the behaviour of pyruvate *added* to the soluble fraction of the Ac-filtrate used for chromatography.

Against all assumptions and known experience in paper chromatography, the Na-salt now located itself *above* the creatinine spot *in one line* with the unknown substance.

This far-reaching influence on the R_F due to the complicated medium of the serum has led us to carry out analogous experiments with hydantoin, methylhydantoin and glycocyamidine.

Chromatography from BuAc-mixture as well as from butanol itself merely supported the results of the preceding experiments on the chromatography of these individual substances.

From this it seems to follow that the case mentioned above can be considered to be an indication of a specific property of pyruvic acid (its sodium salt); the formation of a complex of pyruvic acid with some substance or system of substances in the complex medium of the applied sample cannot be excluded, this complex exhibiting a different R_F. The presumed complex ought to be sufficiently labile to hydrolyse in water, as is indicated by the polarographic investigation discussed below. Another possibility is the change of the partition coefficient of pyruvic acid caused by the presence of other substances.

To prove definitely the identity of the unknown substance with Na-pyruvate, *water eluates* of two untreated spots obtained by chromatography of 75 μl of Ac-filtrate, were examined *polarographically* as well as by *spectrophotometry* of the corresponding 2.4-dinitrophenylhydrazone.

I. POLAROGRAPHY*

The eluate (total volume 0.8 ml) gained in the manner stated above was examined polarographically in a carbonate buffer of p_H 11. The curves were recorded from −0.8 V, at h = 26 cm Hg-level, t_1 = 3.0 sec, 3.8 V accumulator, 1 cm corresponds 0.55 μA.

Fig. 3. Polarography of the unknown component from eluates of spots on chromatograms:–

1. Solution tested
2. Sample + 0.1 ml of solution of sodium pyruvate corresponding to 2.2 μg pyruvic acid
3. Sample + 0.2 ml of solution of sodium pyruvate corresponding to 4.4 μg pyruvic acid

* The polarographic estimations were kindly carried out by P. ZUMAN by his own, still unpublished method.

References p. 222/223.

2. SPECTROPHOTOMETRY[47]

2 ml of the eluate in distilled water was warmed for 20 minutes at 55° C after addition of 0.5 ml of a solution of hydrazine reagent (2.7 g hydrazine hydrochloride + 30 ml water + 100 ml methyl-alcohol + 25 ml 36% HCl) to eliminate ketoglutaric and oxalacetic acids* possibly present. After addition of 1 ml of a 0.1% solution of 2.4-dinitrophenylhydrazine in 2*N*-HCl, the mixture was left to stand for 10 minutes, and shaken with 7 ml of xylene added from a pipette; the xylene extract was then itself shaken with 6 ml of a 10% soda solution, to 5 ml of which was added 5 ml of 2*N*-NaOH and the mixture examined with a COLEMAN spectrophotometer.

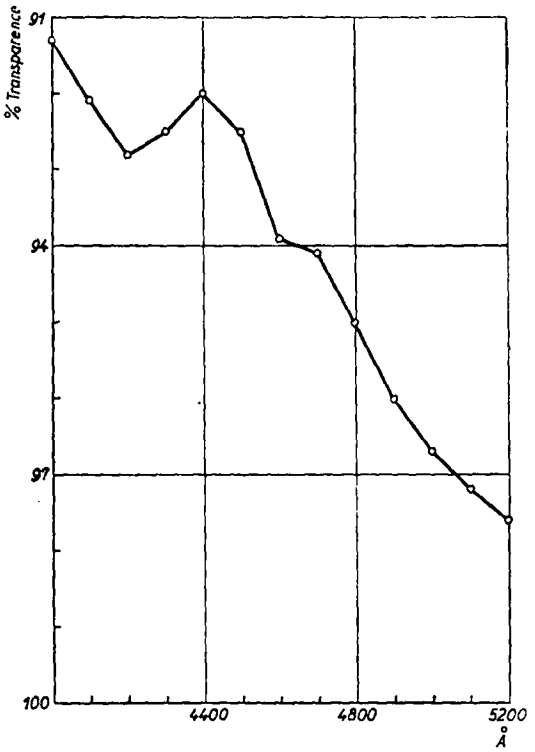

Fig. 4. Spectrophotometry of 2.4-dinitrophenyl hydrazone from eluates of spots of the unknown component on chromatograms

The maximum of absorption of the unknown component (4400 Å) corresponds exactly to maximum of the synthetic Na-pyruvate treated in the same manner.

SUMMARY

1. A method for the detection of Jaffé-positive substances in human blood serum using the technique of *paper partition chromatography* is described.

2. A partial solution of the problem of apparent creatinine has been furnished by identification of *pyruvate* as a second Jaffé-positive compound beside creatinine in a water-soluble fraction of acetone-filtrate of sera; this finding was confirmed *polarographically* as well as *spectrophotometrically*.

Up till now the *quantitative* significance of this substance has not been considered in connection with this problem.

3. After a few preliminary experiments, paper chromatography of the insoluble residue of acetone-filtrate of serum has been left open for further investigation.

* Modification by K. SLAVÍK AND Č. MICHALEC[48].

References p. 222/223.

It seems likely that the final solution of the problem of apparent creatinine will cover *both* possibilities which were discussed in the introduction, irrespectively of whether the chromatographic investigation of the insoluble fraction might show the presence of some new unknown Jaffé-positive component.

RÉSUMÉ

1. Une méthode a été décrite pour la détection de substances dans le sérum sanguin humain donnant une réaction de Jaffé-positive. Elle utilise la technique de la *chromatographie de partition sur papier.*

2. Une solution partielle du problème de la créatinine apparente a été donnée par l'identification de l'acide pyruvique (sel de Na) comme second constituant à réaction de Jaffé positive, à côté de la créatinine, dans la fraction soluble du filtrat à l'acétone du sérum. Cette identification a été confirmée par *polarographie* ainsi que par la méthode de *photométrie spectrale.*

Cette substance n'avait jamais été considérée en relation avec ce problème, du moins en ce qui concerne son importance *quantitative.*

3. Après quelques essais préliminaires, la chromatographie sur papier du résidu insoluble du filtrat à l'acétone du sérum a été laissée de côté pour des recherches ultérieures plus détaillées.

Il semble probable que la solution finale du problème de la créatinine apparente sera fournie par la combinaison *des deux possibilités* envisagées dans l'introduction, quel que soit le résultat des recherches ultérieures sur la présence, dans la fraction insoluble, d'un composé inconnu à réaction de Jaffé-positive.

ZUSAMMENFASSUNG

1. Es wurde eine Methode für die Auffindung von Jaffé-positiven Stoffen in menschlichem Blutserum beschrieben, die die Technik der *Verteilungschromatographie* auf Papier gebraucht.

2. Einen Beitrag zum Problem des scheinbaren Kreatinins bildet die Identifikation der *Brenztraubensäure* (ihres Na-Salzes) als des zweiten Jaffé-positiven Bestandteiles neben Kreatinin in der löslichen Fraktion des Aceton-filtrates vom Blutserum. Dieser Befund wurde durch die *polarographische* und *spektrophotometrische* Untersuchung bestätigt.

Dieser Stoff wurde bisher von keinem der Forscher in Betracht gezogen, die sich mit diesem Problem vom Standpunkte der *quantitativen* Untersuchungen aus beschäftigten.

3. Die Verteilungschromatographie des unlöslichen Rückstandes des Acetonfiltrates wurde nach einigen Vorversuchen zur weiteren ausführlichen Untersuchung offengelassen.

An der vollkommenen Lösung des Problems des scheinbaren Kreatinins werden gewiss *beide* Möglichkeiten teilnehmen, die in der Einleitung besprochen wurden. Dies gilt auch für den Fall, dass die chromatographische Bearbeitung der unlöslichen Fraktion die Anwesenheit eines neuen unbekannten Jaffé-positiven Stoffes anzeigen sollte.

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Received May 5th, 1949