"CHARGE-TRANSFER THIN-LAYER CHROMATOGRAPHY" OF VARIOUS BIOCHEMICALS

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SUMMARY

Thin-layer chromatographic studies have been made of purines, pyrimidines, nucleotides, nucleosides and amino acids with different charge acceptors either mixed together or with the acceptor as part of the stationary phase. These have been complemented with infrared spectrophotometry and fluorescence quenching studies.

INTRODUCTION

"Charge-transfer thin-layer chromatography" has been studied by several authors[1-4] either as a means of obtaining better separation of related compounds or as a technique to detect the formation of charge-transfer complexes. Previous studies have concentrated on organic donors and acceptors in the main. In this work studies on biochemical systems are reported.

Charge-transfer complexes are weak complexes involving electron donors and acceptors. In the ground state of the complex, there is a small amount of charge donated from the donor to the acceptor. On the absorption of light of suitable energy there is a much large donation of charge giving rise to the characteristic charge transfer absorption band and hence colour of the complex. Full discussion of this topic is given in the books of Mulliken and Person[5], Foster[6,7] and Slifkin[8]. The latter author specifically deals with charge-transfer complexes of biomolecules. The role of charge-transfer complexing in biochemical systems is the subject of much discussion and controversy[7,8], and hence analytical methods for proving or confirming the presence (or indeed otherwise) of charge-transfer forces in these systems are of some interest.

Three kinds of experiment have been carried out previously; those in which ready made thin-layer chromatography (TLC) plates are soaked in solutions of the acceptor in order to load them into the stationary phase; those in which plates are made using a slurry of silica gel and acceptor; and chromatography of mixtures of donor and acceptor on standard silica gel plates.

In our own experiments presented here we have utilised the latter two methods plus a more novel technique of covalently binding suitable acceptors to silica gel or cellulose and hence making up to plates.

It is the difference between the R_F values of the donors with and without acceptor which is a measure of the interaction. Harvey and Halonen[1] have proposed the concept of a binding constant B, defined as

$$B = \frac{R_F - R_F'}{R_F} \times 100$$

where R_F is the value obtained in the absence of acceptor and R_F' in the presence of the acceptor. Harvey and Halonen[1] suggest that B is a direct measure of charge transfer interaction. In fact there are difficulties with this B parameter. Literature values are frequently negative owing to the increase of R_F with electron acceptor. Furthermore the B parameter is asymmetric, taking values from $-\infty$ to 100. It is not clear what the relationship is between B and the interaction forces or even whether two identical B values imply forces of the same magnitude if the R_F values are widely different.

EXPERIMENTAL

Chemicals were the purest commercially available and used as supplied, except quinone purified by sublimation and chloranil by recrystallisation. Silica gel used was Kieselgel GF_{254} from E. Merck (Darmstadt, G.F.R.). This contains a fluorescent indicator and was useful for the detection of the nucleic acid bases which showed up as a dark spot against a fluorescent background. The cellulose was MN300 for TLC from Machery, Nagel & Co. (Düren, G.F.R.). TLC plates were made using the Shandon Unoplan apparatus. All plates used were 20×20 cm squares of glass which were first thoroughly cleaned using Decon 75 surfactant. Two different kinds of slurry were made from which TLC plates were obtained. The first consisted of 40 g of silica gel mixed with 80 ml of benzene–95% ethanol (92:8). The second consisted of 40 g of silica gel mixed with 92 ml of distilled water and 8 ml of methanol. The silica gel thickness was set to 25 mm.

When impregnants were used, they were added to the slurry solvent to make up a $5 \cdot 10^{-5}$ M solution. After the slurry was spread on the plates, they were dried and then stored in an oven overnight. Plates containing riboflavin were stored in the dark to prevent photooxidation.

Cellulose plates were made using a slurry of 7 g of cellulose and 30 ml of water with 4 ml of ethanol, and otherwise as for silica gel.

Riboflavin covalently bound to cellulose was made according to the method of Arsenis and McCormick[9].

The silica gel riboflavon covalent compound was prepared using a method due to Mikeš *et al.*[10] for linking silica gel to 2-{(2,4,5,7-tetranitro-9-fluorenylidene)amino}oxy}proprionic acid. The steps in the procedure are shown in Fig. 1. The implementation can be found in ref. 10.

After chromatography amino acids were spotted using ninhydrin solution (2% in acetone) and the purines and pyrimidines were detected by ultraviolet (UV) light (254 nm).

All quoted R_F values are the mean of six readings.

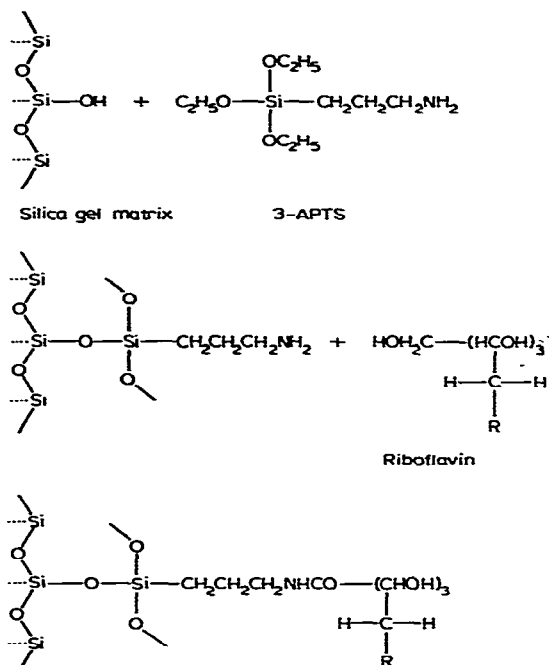Infrared (IR) spectra were obtained using a Unicam SP200G spectrophotome-

Fig. 1. The coupling of riboflavin to silica gel.

ter. Samples of complexes were evaporated from mixed ethanol–water solution in a rotary evaporator and made into KBr discs using standard sampling techniques. A Perkin-Elmer 2000 fluorescence spectrophotometer with constant temperature cell holder was used. All fluorescence quenching studies were carried out in pH 7 phosphate buffer.

RESULTS AND DISCUSSION

TLC of mixed solutions of pyrimidines with different electron acceptors, chloranil, bromanil, trinitrobenzene and tetracyanoethylene on silica gel yielded R_F values no different from those of the pyrimidines alone. IR spectroscopy of evaporates of mixed solutions appear to be the superposition of the IR spectra of the individual components. It can be assumed that in these systems any interaction is very weak.

With purines and the same acceptors there are obvious colour changes which are greatly intensified on freezing down to liquid nitrogen temperatures (colours of evaporates are shown in Table I). This is usually taken to be due to charge-transfer complexing[5–8]. Some of these systems when evaporated display electron spin resonance (ESR) signals as listed in Table II, which is an indication of electron decoupling. Table III lists R_F values of both individual compounds and mixtures with charge acceptors. In almost all cases the R_F values of the mixtures show retardation compared with the individual components, together with streaking between the two spots of the mixture. The streaking would seem to imply that there is some weak interaction in these systems leading to continual formation and dissociation of complexes between the purines and acceptors. The retardation of the spots has been interpreted by

TABLE I

COLOURS OF THE PURINE–ACCEPTOR COMPLEXES

TCNE = Tetracyanoethylene; TNB = 1,3,5-trinitrobenzene.

	TCNE	Chloranil	Bromanil	p–Benzo-quinone	TNB
Uric acid	Yellow	Pale yellow	Light brown	Dirty yellow	Cream
Caffeine	Yellow	Light green	Reddish brown	Brown	Cream
2,6-Diamino-purine	Yellow	Light green	Reddish brown	Brown	Cream
6-Methylamino-purine	Bright yellow	Yellow	Chocolate brown	Chocolate brown	Cream
Adenine	Bright yellow	Dirty green	Light brown	Light brown	Pale yellow
Hypoxanthine	Bright yellow	Dirty green	Chocolate brown	Brown	Buff
Purine	Bright yellow	Pale yellow	Brown	Brown	Pink

Schenk *et al.*[4], who observed similar effects in organic systems, as arising from weak complexing. TLC does appear to give an indication of weak interactions between molecules by comparing the difference between systems and individual components. The pyrimidines, which show no interaction under these conditions, have rather higher ionisation potentials than the purines[11] and are expected consequently to form weaker complexes.

Details of the IR spectra of the evaporates of these systems are given in the Appendix, and they indicate the presence of charge transfer forces between the purines and acceptors.

An alternative method of examining weak interactions is to have one of the interacting components incorporated into the stationary phase of the TLC plate. The method of soaking a silica gel TLC plate in a solution of the impregnant is, we feel, unsatisfactory as one has little control over the amount of impregnant or its distri-

TABLE II

COMPLEXES THAT GAVE ESR SIGNALS

	TCNE	Chloranil	Bromanil	p–Benzo-quinone	TNB
Uric acid	Yes	No	No	Yes	No
Caffeine	Yes	Yes	No	No	No
2,6-Diaminopurine	Yes	No	No	No	No
Hypoxanthine	Yes	No	No	No	No
6-Methylaminopurine	Yes	No	No	Yes	Yes
Adenine	Yes	Yes	Yes	No	No
Purine	Yes	No	No	No	No

TABLE III

R_F VALUES OF PURINES, ACCEPTORS AND THEIR MIXTURES ON SILICA GEL

S = Streaky; BQ = p-benzoquinone. Solvent, chloroform–methanol (4:1).

	TCNE (0.85)	Chloranil (0.82)	Bromanil (0.88)	TNB (0.84)	BQ (0.86)
Uric acid (0.31)	0.28,0.84	0.21,0.74 S	0.26,0.75 S	0.25,0.89	0.29,0.90
Caffeine (0.82)	0.71,0.85	0.82,0.87 S	0.77,0.84	0.69,0.81	0.80,0.85
2,6-Diaminopurine (0.20)	0.16,0.85	0.18,0.83	0.13,0.84	0.14,0.87 S	0.14,0.85
Hypoxanthine (0.20)	0.17,0.85 S	0.17,0.85 S	0.16,0.85 S	0.15,0.85 S	0.18,0.85
6-Methylamino-purine (0.51)	0.40,0.84	0.35,0.83 S	0.36,0.82 S	0.38,0.82 S	0.50,0.84
Adenine (0.30)	0.22,0.85 S	0.24,0.86 S	0.23,0.88 S	0.30,0.84 S	0.26,0.84 S
Purine (0.32)	0.29,0.85 S	0.28,0.82	0.25,0.88	0.26,0.79 S	0.30,0.85

bution through the stationary phase. The method of making up plates with the silica gel mixed with the impregnant is more satisfactory as one can at least have better control over the amount of impregnant and its distribution. Two different sets of experiment are reported, the first in which benzene was used as a binder and the second in which methanol was used. Our original intention was to use only benzene. This gives a very good quality plate with no flaking of the silica gel from the edges.

TABLE IV

R_F VALUES IN IMPREGNATED TLC PLATES; BENZENE BINDER

A = Plain; B = p-benzoquinone; C = trinitrobenzene; D = chloranil; solvent, chloroform–ethanol (4:1). E = p-Benzoquinone; F = trinitrobenzene; G = chloranil; H = plain; I = riboflavin; solvent, butanol–acetic acid–water (12:3:5).

	A	B	C	D	E	F	G	H	I
Caffeine	0.64	0.72	0.67	0.66	0.48	0.58	0.60	0.53	0.50
Uric acid	0.00	0.00	0.00	0.00	0.50	0.00	0.15	0.53	0.00
Adenine	0.24	0.36	0.26	0.35	0.40	0.49	0.53	0.33	0.47
2,6-Diamino-purine	0.14	0.20	0.15	0.00	0.27	0.47	0.49	0.17	0.41
Purine	0.30	0.39	0.32	0.21	0.41	0.51	0.50	0.37	0.00
Uracil	0.26	0.24	0.30	0.35	0.50	0.20	0.54	0.40	0.00
Adenosine	0.00	0.00	0.00	0.00	0.00	0.54	0.11	0.30	0.12
Thymine	0.31	0.34	0.40	0.28	0.58	0.42	0.56	0.50	0.58
Thymidine	0.24	0.21	0.30	0.34	0.40	0.31	0.55	0.44	0.52
Hypoxanthine	0.16	0.15	0.19	0.10	0.46	0.00	0.46	0.31	0.17
Gly					0.12	0.12	0.20	0.19	0.26
Ala					0.15	0.22	0.20	0.21	0.27
Pro					0.11	0.18	0.19	0.20	0.25
Phe					0.22	0.42	0.39	0.15	0.50
Try					0.25	0.52	0.45	0.27	0.56

However, the introduction of new safety rules in the University during the course of these experiments forbade the further use of benzene; the experiments were then continued using methanol.

The next set of experiments to be reported used benzene as a binder. R_F values for a variety of purines, pyrimidines and amino acids are given in Table IV. The corresponding B values are given in Table V.

TABLE V

B VALUES FOR IMPREGNATED TLC PLATES; BENZENE BINDER

Columns as in Table IV.

	B	C	D	E	F	G	I
Caffeine	−13	− 5	− 3	9	− 9	− 13	6
Uric acid	0	0	0	6	100	72	100
Adenine	−50	− 8	− 46	− 21	− 48	− 61	− 47
2,6-Diaminopurine	−43	− 7	100	− 58	−176	−188	−141
Purine	−30	− 6	30	− 11	− 80	− 35	100
Uracil	8	− 5	− 35	− 25	50	− 35	100
Adenosine	0	0	0	100	− 80	63	60
Thymine	−10	−29	10	− 16	16	− 12	− 16
Thymidine	13	−25	− 42	9	29	− 25	− 18
Hypoxanthine	6	−19	38	− 48	100	− 48	45
Gly				37	58	− 5	− 37
Ala				27	− 5	5	− 28
Pro				45	10	5	− 19
Phe				− 46	−180	−160	−233
Try				7	− 93	− 67	−107

Of the purines, 2,6-diaminopurine gives the biggest changes in R_F, i.e. the biggest B values. This compound, with its two amino groups, is expected to be the strongest charge donor among this group. However, this is not true for all the acceptors used. Similarly, in general the B values for the acceptors go in the order chloranil > trinitrobenzene > quinone in chloroform–methanol, and in exactly the reverse order in butanol–acetic acid–water. Chloranil forms stronger charge transfer complexes than trinitrobenzene, which in its turn is stronger than quinone[6]. The polarity of the solvent is clearly a determining factor in the interaction. Those purines that do not move during TLC are the very insoluble ones. In these experiments the pyrimidines do exhibit some interaction with the acceptors, thus showing this to be a more sensitive technique.

There is an interesting phenomenon among the amino acids in that the B values for the aromatic amino acids are much greater than those of the aliphatic amino acids for most acceptors, indicating the involvement of the pi-electrons in these interactions. There is no sign of new chemical compounds found with the acceptors, as suggested by Foster[7]. B values take both positive and negative values. Negative values have been explained by Schenk et al.[4] as being due to masking of active hydroxyl sites of silica gel by the acceptor. This is not a wholly satisfactory explanation, particularly as it can be easily demonstrated that in many of the systems studied, some of the impregnated acceptor itself moves up the plate with the solvent.

This is particularly noticeable when riboflavin is used, as the distribution of fluorescence after chromatography is changed even with no donor. These negative B values can arise by the impregnant actually dragging the donor with it up the plate and hence the largest negative values imply greatest interaction.

Although these experiments point to there being some correlation between charge transfer parameters and changes in R_F, the matter is by no means clear cut. The solvent used and even the binder, as will be subsequently shown, are as important if not more so in determining the interaction. However, one can clearly detect that these are weak interactions rather than strong chemical ones.

In view of the unsatisfactory nature of just mixing the acceptor with the silica gel, another technique has been tried in which roboflavin was covalently bound to silica gel or to cellulose. All these experiments were carried out using methanol as a stationary phase binder.

The results are shown in Table VI with corresponding B values in Table VII. The results with cellulose gave in all cases extremely small R_F values, which would suggest that there is very strong binding between these amino acids and riboflavin in this system. In these experiments, unlike the previous, there are no differences between aliphatic and aromatic amino acids indicating that in these systems it is the lone-pair electrons on the amino group of the amino acids which are primarily participating in the interaction. It will seem that in this system the π-electron system of the aromatic amino acids is unable to orient itself favourably with the acceptor. In view of the experimental difficulties of preparing cellulose-bound riboflavin and making good quality plates, it was decided to concentrate on riboflavin-bound silica gel, the results of which are also shown in Tables VI and VII.

TABLE VI

R_F VALUES IN IMPREGNATED PLATES; METHANOL BINDER

A = Plain; B = impregnated riboflavin; C = cellulose; D = riboflavin bound cellulose; E = 5% (w/w) bound riboflavin; F = 0.05% (w/w) bound riboflavin; G = 0.0125% (w/w) bound riboflavin; all with butanol–acetic acid–water (12:3:5) and silica gel, except for C and D.

	A	B	C	D	E	F	G
Phe	0.47	0.53	0.73	0.10	0.35	0.39	
Ile	0.44	0.50	0.80	0.11	0.36	0.35	
Tyr	0.43	0.50	0.54		0.36		
Ser	0.22	0.26	0.22	0.04	0.15	0.15	
Pro	0.22	0.22	0.45	0.04			
Glu	0.29	0.31	0.25	0.02	0.19	0.19	
Asp	0.25	0.27			0.12	0.12	
Ala	0.30	0.30			0.19	0.20	
Trp	0.43				0.33		
Adenosine	0.44				0.45	0.40	0.42
AMP	0.16				0.08	0.08	0.10
ADP	0.05				0	0.03	0.02
ATP	0.02				0	0.02	0
c-AMP	0.20				0.15	0.12	0.17
Cytosine	0.29				0.27	0.26	0.24
Thymine	0.61				0.57	0.51	0.54
Uracil	0.54				0.53	0.47	0.52
Adenine	0.45				0.45	0.38	0.43

TABLE VII

B VALUES FOR TLC PLATES USING METHANOL AS BINDER

Columns as in Table VI.

	B	*D*	*E*	*F*	*G*
Phe	−13	86	26	17	
Ile	−14	86	18	20	
Tyr	−16		16		
Ser	−18	82	32	32	
Pro	0	91			
Glu	− 7	92	35	35	
Asp	− 8		32	52	
Ala	0		37	33	
Trp	0		23		
Adenosine			−2	9	5
AMP			50	50	38
ADP			100	40	60
ATP			100	0	100
cAMP			25	40	15
Cytosine			7	10	17
Thymine			7	16	11
Uracil			2	13	4
Adenine			0	16	4

A novel set of experiments has been to use different ratios of bound silica gel to unbound silica gel, to see whether masking effects can be detected. In fact there are no significant differences between different amounts of acceptor, so that in these systems at least masking does not occur. Furthermore, with bound acceptors there are no negative *B* values, reinforcing the view that negative *B* values arise from the impregnant itself moving through the stationary phase dragging the donor with it.

Among the results shown in Tables VI and VII, are those for a group of nucleotides and nucleic acid bases. Adenosine, uracil, cytosine and adenine show little sign of interaction, unlike AMP, ADP, ATP and cAMP. It can be seen with this group of compounds that the presence of additional phosphate groups lowers the R_F value on plain silica gel according to the number of phosphate groups available. Interestingly cAMP, in which the phosphate group is less available for interaction, has a smaller R_F value than AMP on plain silica gel and also appears to be slightly less inhibited on the riboflavin bound silica gel. From these observations, it can be concluded that the major interaction with the riboflavin occurs through the phosphate group and that therefore there is competition between the riboflavin and silica gel for interaction with phosphate group.

Tsibris *et al.*[12] have suggested that the addition of a phosphate group to the adenosine molecule inhibits complex formation with riboflavin as indicated by fluorescence quenching studies. This is exactly the reverse of what is reported here. We have also carried out some fluorescence quenching experiments on these systems. Stern–Volmer plots are shown in Fig. 2 and dissociation constants therefrom in Table VIII. The values for AMP and adenosine agree with those of Tsibris *et al.*[12]. Those for ATP and cAMP are new. The dissociation constant for cAMP with its less readily available phosphate group is identical with that of the unphosphorylated adenosine
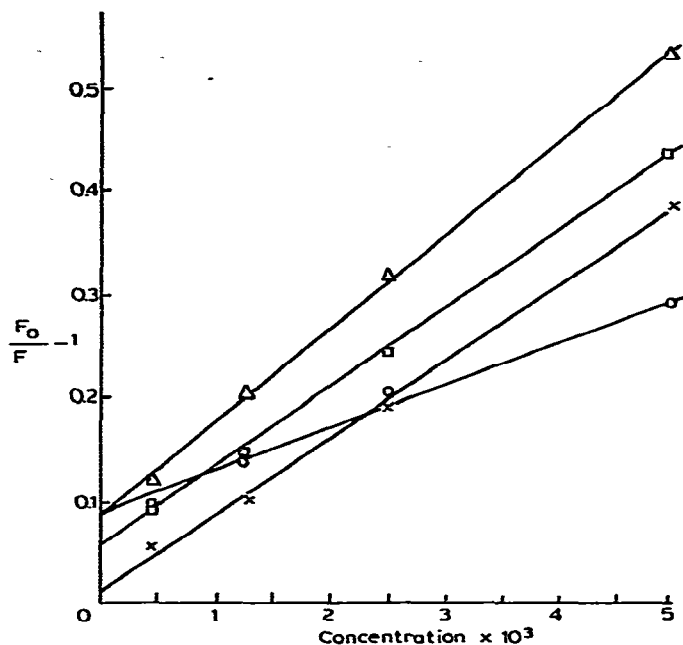
Fig. 2. Stern–Volmer plot showing the quenching of riboflavin fluorescence by adenosine (×), AMP (O), c-AMP (△) and ATP (□) at pH 7, and room temperature. Riboflavin concentration, $5 \cdot 10^{-3}$ M.

whereas that for AMP with its free phosphate group is higher and that for ATP lower. Thus there is no correlation between R_F or B values and fluorescence quenching, and the suggestion of Tsibris et al.[12] appears to be incorrect. The interaction involved in fluorescence quenching is not the same as that involved in TLC with riboflavin.

TABLE VIII

DISSOCIATION CONSTANTS OBTAINED FROM STERN–VOLMER PLOTS FOR THE QUENCHING OF RIBOFLAVIN BY NUCLEIC ACID BASES AND NUCLEOTIDES IN pH 7 PHOSPHATE BUFFER

r is the correlation coefficient in the linear regression analysis for the Stern–Volmer plots.

	Adenosine	AMP	ATP	cAMP
K_{diss} ($\times 10^3$)	13.6	25.6	11.0	13.7
r	0.995	0.998	0.9995	0.999

An alternative form of charge-transfer chromatography is column chromatography using either molecular-sieving gels (Sephadex)[13–17] or silica gel matrices[18]. Thus Porath and Larsson[13] have looked at the chromatography of amino acids on Sephadex gels cross-linked to a variety of electron acceptors and shown that weak interactions including charge transfer occur between the amino acids and the acceptors. Similar results have been found from the chromatography of nucleotides on Sephadex linked to the acceptor acriflavin. In particular, it is shown that the purines

interact more strongly than pyrimidines[14], paralleling our results with TLC. Column chromatography on silica gel mixed with riboflavin has been found to give separation of enantiomeric forms of carbohelicenes by what is presumed to be charge-transfer interaction[18].

These different forms of column chromatography are more complex, expensive and time-consuming than TLC but probably give better resolution. These methods are complementary, in giving similar results, with the TLC method being useful for rapid and inexpensive screening of systems.

In conclusion, weak interactions in biochemical systems can be detected by different forms of "charge-transfer TLC". There is some correlation between parameters associated with charge transfer complexes and B values. Other factors, such as the method of preparation of the plate and solvent polarity, are also important in determining the interaction. The method of covalently binding the acceptor to the stationary phase is, we feel, a better method for these studies than just mixing the acceptor with the silica gel as it guards against migration of the acceptor.

APPENDIX

This is a very brief survey of the IR spectra obtained from evaporates of equimolar solutions.

(A) The purine chloranil evaporates exhibit the following common features:

(1) Shift of C–O stretch of chloranil from *ca.* 1690 to *ca.* 1670 cm^{-1}.

(2) Weakening of C–Cl stretch of chloranil at 725 cm^{-1}.

(3) Decrease of N–H stretching frequencies at 3300 cm^{-1}.

(4) Decrease of N–H deformation at 1610 cm^{-1}.

(5) Decrease of C–N stretching frequencies at 1310 cm^{-1}.

(6) Suppression of C–C (aromatic) stretches at 1580 cm^{-1}.

Purine bromanil evaporates are similar to the above showing features 1,3,6 and in addition.

(7) Weakening of C–Br stretch at 730 cm^{-1}.

Purine quinone evaporates are similar to the above with features 1 and 6, together with decrease of intensity of many stretching frequencies.

(B) Purine trinitrobenzene evaporates exhibit the following common features:

(1) Decrease and shift of C–C (aromatic) stretching frequencies to *ca.* 1540 cm^{-1}.

(2) Shift and decrease in intensities of trinitrobenzene stretches
(i) shift of stretch due to trinitro substitution (aromatic) from 1100 to 1180 cm^{-1};
(ii) shift of symmetric nitro stretches from 1380 to 1340 cm^{-1}.

(C) Purine TCNE spectra obtained from well-ground equimolar mixtures exhibit the following common features:

(1) New slight band at *ca.* 1530 cm^{-1}.

(2) Decrease in C–C (aromatic) stretch frequencies.

(3) Decrease in N–H deformation at 1620 cm^{-1}.

(4) Decrease in C–N stretching frequencies at 1280 and 1350 cm^{-1}.

All the above are consistent with the formation of charge-transfer complexes[19,20].

Evaporates of purine TCNE solutions give off a greenish blue vapour leaving a

bright yellow residue, except for caffeine and uric acid which leave behind a tarry substance. From IR and UV-visible spectroscopy it would appear that the yellow substance contains the 1,1,2,3,3,-pentacyanopropenide anion and the black residue is a tricyanoethanol.

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