SUMMARY

1. The haemolymph and the green parts of the integument of solitary locusts contain typical insectoverdins which can be resolved into yellow and blue components which are chromoproteins.

2. The prosthetic group of the blue chromoprotein is in each case probably mesobiliverdin. The yellow component of the haemolymph is a β-carotene-protein complex whilst that of the integument contains both β-carotene and free astaxanthin; this probably means the co-existence of two yellow chromoproteins in the integument, although the possibility of both carotenoids being attached to the same protein has not been ruled out.

3. A small amount of a yellow water-soluble pterin-like material also occurs in the haemolymph and integument; its contribution to the green coloration can only be slight.

4. Neither astaxanthin nor insectorubin occurs in haemolymph. Both are therefore probably synthesized in the integument, the former from β-carotene and the latter from unknown precursors.

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Derivatives of Adrenaline and Noradrenaline in an Extract of an Adrenal Medullary Tumour

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Although pharmacological evidence of the occurrence of noradrenaline in the body has been accumulating since Barger & Dale's (1910) classical work on the sympathomimetic amines (for references, see Bergström, Euler & Hamberg, 1950), the first unequivocal demonstration of its presence in the animal body was made by Holton (1949). Using a paper chromatographic technique devised by James (1948), Holton found that the main pressor component of extracts of adrenal medullary tumours was noradrenaline, adrenaline also being present but in much smaller quantity. This finding has been confirmed by Goldenberg, Faber, Alston & Chargaff (1949), who further showed the presence of noradrenaline in U.S.P. adrenaline prepared from cattle suprarenals. Euler & Hamberg (1949) have also demonstrated chemically the occurrence of noradrenaline, in addition to adrenaline, in extracts of cattle suprarenals. Moreover Bergström *et al.* (1950) and Tullar (1949) have isolated it from this source.

Through the kindness of Prof. G. W. Pickering, of St Mary's Hospital, London, the author had the opportunity of examining an extract of a human adrenal medullary tumour by paper chromatography. Adrenaline, noradrenaline, and a number of related compounds may be detected on the developed chromatogram, by spraying the dried paper with a solution of potassium ferricyanide in phosphate buffer pH 7·8 (James, 1948). With this reagent the compounds yield red or purplish-red derivatives. A chromatogram of the tumour extract, on spraying with the ferricyanide reagent, showed the presence of four substances yielding red-coloured derivatives. Of these, two occupied positions which corresponded

with those of noradrenaline and adrenaline. Further investigation indicated that the other two substances were derivatives of adrenaline and noradrenaline. Isolation of these substances for complete chemical characterization was not possible through lack of material. Evidence was obtained, however, which pointed to the probability of their being esters of the amines formed by the union of the secondary alcoholic group on the side chain with the carboxyl group of lactic acid.

As far as is known, the only report in the literature of such a type of compound being present in biological material has been that of Kendall (1932), who found in an extract of adrenal glands a substance which he considered to be lactyladrenaline. In the more recent investigations on extracts of adrenal glands and adrenal medullary tumours (Holton, 1949; Euler & Hamberg, 1949; Goldenberg *et al.* 1949) in which the presence of adrenaline and noradrenaline was demonstrated by paper chromatography using the ferricyanide reagent, no evidence of any other similarly reacting compound was obtained. In view of this, it was necessary to consider the possibility that the additional compounds detected by the author did not actually occur as such in the tumour and appeared in the final preparation as artifacts of the extraction procedure. Further experiments showed that such was indeed highly probable. The author, however, feels that these findings should be communicated if only for the reason that they demonstrate the danger of the possible formation of artifacts in the preparation of biological materials. In addition, it is not inconceivable that these esters may have some biological significance in view of their relative ease of formation and of breakdown, and also of their relative lack of pharmacological actiyity when compared with the parent amines.

EXPERIMENTAL

Preparation of extract of adrenal medullary tumour

The adrenal medullary tumour was received in small pieces immersed in 0·1 N-HCl (10 ml./g.). The tissue and the brown fluid were ground together in a glass mortar with a little acid-washed silver sand. The extract was centrifuged and the supernatant adjusted to pH 4 (glass electrode) by the addition of 2 N-NaOH. To remove protein, the solution was poured into 15 vol. aldehyde-free ethanol containing 0·05 ml. conc. HCl (sp.gr. 1·18)/100 ml. After standing at approx. 0° for 2 hr., the solution was freed from precipitated protein by centrifugation and evaporated to dryness *in vacuo* at 60–65°. The reddish-brown residue was taken up in 0·01 N-HCl (1 ml./0·5 g. tumour). Some reddish-brown insoluble material was centrifuged off to give the yellow, faintly opalescent solution which was used in the experiments described below.

Paper chromatograms

All chromatograms were carried out by the capillary ascent method of Williams & Kirby (1948). A sheet of Whatman no. 1 filter paper (37 × 39 cm.) was fashioned into cylindrical form by joining the longer sides edge to edge with cellulose tape. Test solutions were applied as single or replicate drops, each 5 μl., to the paper at a distance of 5 cm. from one end of the cylinder and at least 6 cm. away from the joined edges, since the solvent flow tends to be irregular near the join. After the test solutions had been allowed to evaporate on the paper at room temperature, the paper cylinder was stood in a glass tank (15 × 32 × 45 cm.) the foot of which contained the developing solvent (500 ml.) to a depth of about 1·5 cm. The tank was closed by a vaselined glass plate which carried an inlet tube for the introduction of SO_2. After development of the chromatogram for 20–24 hr., during which time the solvent had risen some 30–34 cm., the solvent was removed from the paper by drying in an oven at 40° for 3 hr., a current of N_2 being passed through the oven to facilitate removal of the solvent. The dried paper was then sprayed with 0·44 % (w/v) $K_3Fe(CN)_6$ in 0·2 M-phosphate buffer pH 7·8 (James, 1948). Adrenaline and certain related substances are thus rendered visible in the form of red or reddish-purple oxidation products. Quantities of adrenaline and noradrenaline down to 5 μg. may be detected on the paper in this way.

Water-saturated phenol was used in all but one of the chromatograms as developing solvent. The phenol was first distilled from zinc dust at atmospheric pressure, a procedure recommended by Williams & Kirby (1948). These chromatograms were carried out in an atmosphere of SO_2, the gas being passed into the tank for some 10–15 min. after the introduction of the paper. By this procedure, the solvent was rendered strongly acidic and also strongly reducing and oxidation of the catechol amines was prevented or diminished. The use of SO_2 in this way was incidental to the development of a method for the quantitative separation of adrenaline and noradrenaline. *n*-Butanol saturated with 0·5 N-HCl was used as developing solvent on one occasion. The alcohol had first been rendered aldehyde-free by refluxing with NaOH and distilling.

RESULTS

Paper chromatograms of the tumour extract

Paper chromatograms using water- and SO_2-saturated phenol as solvent showed the presence in the tumour extract of four substances yielding red or purplish-red derivatives with the ferricyanide reagent. A rough estimation from the size and colour intensity of the spots suggested that the four substances were present in about equal proportions. The

Table 1. R_F *values* of the four substances present in adrenal-medullary tumour extract using water- and* SO_2*-saturated phenol or* n*-butanol saturated with* 0·5 N-HCl *as solvents*

	R_F (phenol)	R_F (butanol)
Adrenal medullary tumour extract:		
Noradrenaline	0·27	0·14
Adrenaline	0·50	0·19
Compound *X*	0·62	0·55
Compound *Y*	0·75	0·58

* R_F value is the ratio distance of substance from starting point/distance of solvent front from starting point, and is here calculated on the distance from the starting point to the mid point of the spots.

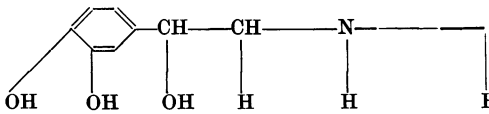
position on the chromatogram of the compound with the slowest flow rate corresponded to that of noradrenaline while the position of the next line corresponded to that of adrenaline. Beyond the adrenaline position lay compound *X*, while still nearer to the solvent front lay compound *Y*. The spots of compound *X* and compound *Y* were V-shaped, indicating retardation of the flow of the main bulk of the material for some reason. Further reference will be made to this later. In Table 1, estimates of the R_F values of the four substances are given.

Comparison of the R_F *values of compounds* X *and* Y *with those of substances related to adrenaline and noradrenaline.* The possibility was first considered that compounds *X* and *Y* might be precursors of adrenaline or noradrenaline or some abnormal metabolites arising in the tumour issue. In an endeavour to identify them, the tumour extract was chromatographed in parallel with a number of possible candidates. The results for this chromatogram are given in Table 2. Reference to this table will show that a number of the substances had R_F values approximating to those of compounds *X* and *Y*. However, on repeating the chromatogram, but using *n*-butanol saturated with 0·5 N-HCl for irrigation of the paper, it was evident that compounds *X* and *Y* were not to be identified with any of the other substances.

Evidence in favour of compounds X *and* Y *being derivatives of noradrenaline and adrenaline.* In the chromatogram run in *n*-butanol, adrenaline and noradrenaline in the tumour extract gave a combined spot paralleled in appearance and position by a mixture of adrenaline and noradrenaline chromatographed simultaneously. The lower half of the spot was coloured purplish-red and the upper half rose-pink as a result of the treatment with the ferricyanide reagent. The fact that compounds *X* and *Y* also gave a combined spot exactly similar in appearance to that of adrenaline and noradrenaline made it probable that compounds *X* and *Y* were similar derivatives of the two amines.

Evidence in favour of compounds X *and* Y *being esters of noradrenaline and adrenaline with the hydroxyl group of the side chains being involved.* The appearance of red-coloured pigments on treating adrenaline (I) or noradrenaline with the ferricyanide reagent is presumably the result of adrenochrome (II) and of noradrenochrome formation (see Bacq, 1949). The production of these compounds involves (*a*) oxidation of the catechol group to a quinone, and (*b*) formation of an indole nucleus by reaction of the benzene ring with the nitrogen atom of the side chain. Since compounds *X* and *Y* both yielded red pigments with the ferricyanide reagent it was therefore concluded, assuming the correctness of the hypothesis of their being adrenaline and noradrenaline derivatives, that the catechol group was still present and that the nitrogen atom of the side chain possessed a hydrogen atom since substitution in this latter position in the case of adrenaline would result in the production of a compound which could not form an indole derivative, as witness the fact that *N*-methyladrenaline did not give a red colour with the ferricyanide reagent (Table 2).

Table 2. R_F *values of adrenaline and related compounds using water- and* SO_2*-saturated phenol or* n*-butanol saturated with* 0·5 N-HCl *as solvents*

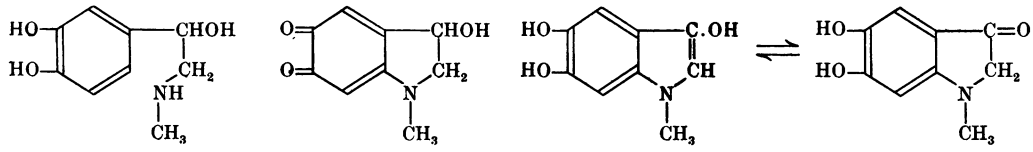
Substance	Formula						R_F Phenol	R_F Butanol
								
Noradrenaline	OH	OH	OH	H	H	H	0·28	0·10
3:4-Dihydroxyphenylalanine	OH	OH	H	COOH	H	H	0·29	0·21
α-Ethylnoradrenaline	OH	OH	OH	C_2H_5	H	H	0·37	0·25
Hydroxytyramine	OH	OH	H	H	H	H	0·43	0·24
Corbasil	OH	OH	OH	CH_3	H	H	0·50	0·33
Adrenaline	OH	OH	OH	H	CH_3	H	0·51	0·17
Isoprenaline	OH	OH	OH	H	$CH.(CH_3)_2$	H	0·66	0·37
Epinine	OH	OH	H	H	CH_3	H	0·67	0·30
N-Ethylnoradrenaline	OH	OH	OH	H	C_2H_5	H	0·68	0·24
Adrenalone	OH	OH	=O	H	CH_3	H	0·69	0·24
N-Methyladrenaline	OH	OH	OH	H	CH_3	CH_3	0·72 (bleached spot no red coloration with the ferricyanide reagent)	0·16
Sympatol	OH	H	OH	H	CH_3	H	Not visible	Not visible
Metasympatol	H	OH	OH	H	CH_3	H	Not visible	Not visible

The evidence already discussed made it likely that compounds *X* and *Y* contained a free catechol group. Support for this belief was obtained from the following experiment.

A chromatogram of the tumour extract was developed in water- and SO_2-saturated phenol and dried thoroughly to remove all the phenol. It was then sprayed with an aqueous

solution of ferric chloride, a reagent which gives a green colour with catechols. The appearance of four green spots on the paper in the positions occupied by the four red spots obtained when the paper was sprayed with the ferricyanide reagent was indicative that not only adrenaline and noradrenaline, but also compounds *X* and *Y* contained a catechol group.

Thus it appeared probable that, of the functional groups of the adrenaline and noradrenaline molecules, the side chain hydroxyl group was the most likely point of substitution in the formation of compounds *X* and *Y*. Further presumptive evidence of this lay in the observation that, if the chromatogram of the tumour extract, after spraying with the ferricyanide reagent, was examined under ultraviolet light, a green fluorescence was apparent only in the adrenaline and noradrenaline positions, no such fluorescence being evident at the positions occupied by compounds *X* and *Y*. According to Lund (1949), the fluorescent substance obtainable from adrenaline results from an intramolecular rearrangement of adrenochrome (II) to give adrenolutine (III and IV) which may exist partly in the enol (III) and partly in the keto form (IV) (Fig. 1). Such tautomerism



Adrenaline (I) | Adrenochrome (II) | Adrenolutine (III) enol form | Adrenolutine (IV) ketoform

Fig. 1.

would not be possible if the side chain hydroxyl group of adrenaline were substituted in any way, and thus it might be expected that no fluorescent compound would be obtained from an adrenaline derivative in which the side chain hydroxyl was, for example, esterified. If compounds *X* and *Y* were indeed esters of noradrenaline and adrenaline, acid hydrolysis should yield the parent amines. This was found to be so.

Acid hydrolysis of compounds X *and* Y

Compounds *X* and *Y* were separated from adrenaline and noradrenaline by paper chromatography. 0·2 ml. tumour extract was applied as a series of 5 μl. drops 5 cm. from the end of a cylinder of Whatman no. 1 filter paper. The chromatogram was developed in water-saturated phenol in an atmosphere of SO_2 for 24 hr. After drying off the solvent a narrow strip was cut from the paper in the direction of the solvent flow. Spraying this strip with the ferricyanide reagent located compounds *X* and *Y* and the corresponding portions were cut from the main bulk of the paper. The materials in the two paper strips thus obtained were eluted with 0·01 N-HCl using a method suggested by Dent (1947). One end of each paper strip was sandwiched between two microscope slides held together by an elastic band. The far sides of the slides were immersed in the elution fluid which passed by capillarity between the microscope slides and then on to and down the paper, the fluid dropping from the end of the paper strip being collected in a test tube. The set-up was covered by a glass tank to prevent undue evaporation of fluid from the paper surface. Elution was allowed to proceed overnight. The eluates were extracted with 3 equal vol. of benzene to remove traces of phenol and evaporated to dryness *in vacuo*. Each residue was dissolved in 0·5 ml. 0·01 N-HCl (unhydrolysed preparation). To effect hydrolysis 0·2 ml. of each solution thus obtained was placed together with 0·2 ml. 2 N-HCl in a small tube which was evacuated, sealed and heated in a boiling-water bath for 30 min. (hydrolysed preparation).

The hydrolysed and unhydrolysed preparations from compounds *X* and *Y*, together with the original tumour extract for comparison, were chromatographed on paper using water- and SO_2-saturated phenol as solvents. Spraying of the developed and dried chromatogram with the ferricyanide reagent gave the following results. From the hydrolysed preparation of *X* a red spot was obtained in a position corresponding with that of noradrenaline. No other spot was visible. The unhydrolysed preparation of *X* gave two spots, one in the noradrenaline position and the other at a position with an R_F value of 0·73. The hydrolysed preparation of *Y* gave only one spot, in the adrenaline position, while the unhydrolysed preparation yielded two spots, the first in the adrenaline position, and the second in a position with an R_F value of 0·90.

It was evident from this experiment that compound *X* was a derivative of noradrenaline and compound *Y* a derivative of adrenaline. Their behaviour to acid hydrolysis was consistent with the hypothesis of their being esters. The presence of the parent amines in the unhydrolysed preparations of compounds *X* and *Y* was probably to be explained by a partial hydrolysis occurring during the evaporation of the acid eluates from the paper strips. It will be noted that the R_F values of compounds *X* and *Y* in the unhydrolysed preparations were somewhat higher than those found when the tumour extract was chromatographed (see Table 1). It appeared likely that the preliminary chromatogram used to separate the compounds from the tumour extract had removed, partially or completely, material which retarded the flow of these substances. The outlines of the spots obtained from the unhydrolysed preparations of compounds *X* and *Y* were more or less oval and not V-shaped as they were when the

tumour extract was used. It was subsequently discovered that a similar increase in the R_F values of compounds *X* and *Y* could be effected in another way.

Separation of compounds X *and* Y *from adrenaline and noradrenaline by use of* n*-octyl alcohol.* In the course of the preparation of one batch of tumour extract, 0·5 ml. *n*-octyl alcohol was added to overcome troublesome frothing encountered during the evaporation of the acid alcohol solution *in vacuo*. The final extract contained two layers, an upper brown octyl alcohol layer and a lower yellow aqueous layer, the former being found to contain the compounds *X* and *Y* and the latter the adrenaline and noradrenaline. Compounds *X* and *Y* could be transferred to aqueous solution by dilution of the octyl alcohol with ether or benzene and extracting with 0·01 N-HCl. A chromatogram of such a solution gave R_F values for compounds *X* and *Y* of 0·79 and 0·89 respectively.

The probable structure of compounds X *and* Y

The question now arose as to what was the exact chemical composition of compounds *X* and *Y*. Unfortunately, adequate characterization of the pure substances was not possible since lack of material precluded their isolation. As a candidate for the position of the esterifying carboxylic acid, lactic acid came first to mind in view of Kendall's (1932) report of the presence of lactyladrenaline in extracts of adrenal glands. Kendall, however, stated that, 'no method was found to liberate adrenaline by hydrolysis'. This was not true of compound *Y*. The presence of a lactyl group in compounds *X* and *Y* might be demonstrated in two ways, (*a*) by preparing the lactyl derivatives of adrenaline and noradrenaline and showing that they behaved in a manner similar to that of compounds *X* and *Y* on a paper chromatogram and (*b*) by testing for lactic acid in the hydrolysed preparations of compounds *X* and *Y*.

Preparation of lactyladrenaline and lactylnoradrenaline. These were prepared according to a method outlined by Kendall (1932) for the preparation of lactyladrenaline. To about 5 mg. (−)-adrenaline or its equivalent of (±)-noradrenaline hydrochloride in a small dry glass tube were added 0·2 ml. methyllactate and a minute drop of conc. H_2SO_4 from a capillary pipette. The tubes were evacuated, sealed and heated in a boiling-water bath for 30 min. After cooling, the tubes were opened and the contents diluted with an equal volume of water.

Comparison of the R_F *values of lactyladrenaline and lactylnoradrenaline with those of compounds X and* Y. From each of the lactyl preparations two red spots were obtained on chromatography on paper with water- and sulphur dioxide-saturated phenol and subsequent spraying of the dried chromatogram with the ferricyanide reagent. In neither case was there any evidence of the presence of the parent amine. The R_F values of the two materials in the adrenaline preparation were 0·82 and 0·92 and of those in the noradrenaline 0·61 and 0·79. In explanation of the occurrence of two substances in these preparations it is tentatively suggested that one might have been an ester of lactic acid while the other was a derivative of lactyl-lactic acid. Whether this explanation be correct or not, the esterification of the amines had resulted in the formation of compounds which behaved on paper in a manner similar to compounds *X* and *Y* (R_F 0·79 and 0·89 respectively) obtained by the octyl alcohol method of extraction described above.

Demonstration of the presence of a lactic acid moiety in compounds X *and* Y. Lactic acid in solution may be detected by the reddish-violet colour which is obtained by the interaction of *p*-hydroxydiphenyl and conc. sulphuric acid with the acetaldehyde resulting from the treatment of lactic acid with hot concentrated sulphuric acid (Feigl, 1947).

To test for the presence of a lactyl radical in compounds *X* and *Y* the following experiment was performed. The compounds were separated from adrenaline and noradrenaline in 0·4 ml. tumour extract by paper chromatography as described above. The eluates containing *X* and *Y* were combined as were those containing the adrenaline and noradrenaline in order to have sufficient material to give an unequivocal result. The combined eluates were evaporated *in vacuo* to a volume of about 1 ml. Conc. HCl (0·1 ml.) was added to each and the solution heated under reflux for 30 min. to effect hydrolysis. The solutions were then further evaporated *in vacuo* to approximately 0·1 ml. and tested for lactic acid. Two drops of each test solution were heated with 1 ml. conc. H_2SO_4 in a dry test tube at 85° for 2 min. After the tubes had been cooled to about 30° under the tap, a small amount of solid *p*-hydroxydiphenyl was added to each solution which was then gently shaken and allowed to stand. A parallel test with pure lactic acid was also performed. On examining the tubes 30 min. later, positive reactions were observed in the tube containing lactic acid, and in the tube containing the hydrolysate from the preparation of compounds *X* and *Y*. The reaction in the 'adrenaline-noradrenaline' tube was negative.

These results were, therefore, indicative of the presence of a lactic acid moiety in compounds *X* and *Y*. This evidence is not conclusive, however, since pyruvic acid and α-hydroxybutyric acid also give a positive reaction (Feigl, 1947).

Compounds X *and* Y *as artifacts of the extraction technique*

Apart from Kendall's (1932) communication, there has apparently been no report in the literature of the occurrence of adrenaline or noradrenaline in the body in compound form of the type exemplified by lactyl-adrenaline. The possibility, therefore, of the formation of compounds *X* and *Y* in the course of the preparation of the extract of the tumour had to be investigated. Indicative that such was the case was the fact that, if the acid-alcohol extract of the tumour was evaporated to a small volume but not to dryness, the fluid contained only adrenaline and noradrenaline. Further evidence of artifact forma-

tion was provided by the observation that the solution obtained from a mixture of adrenaline and lactic acid put through precisely the same procedure as used in the preparation of the tumour extracts, contained, in addition to adrenaline, a substance which behaved on a paper chromatogram in a manner exactly similar to compound *Y* of the tumour extract. Thus, given the presence of adrenaline and lactic acid in the tumour, the indications are that the effect of the extraction procedure would be to produce some of compound *Y*. Noradrenaline might be expected to behave similarly.

Some observations on the pharmacological activities of compounds X *and* Y *and lactyladrenaline and of their hydrolysis products*

The pharmacological activity of these preparations were compared using the rat uterus test described by de Jalon, Bayo & de Jalon (1945) as modified by Gaddum, Peart & Vogt (1949) and by Gaddum & Lembeck (1949). Contraction of rat uterus, suspended in a small bath containing modified Ringer-Locke solution, is produced every 2 min. by the addition of a choline ester (acetylcholine or carbaminoylcholine). The addition of adrenaline or noradrenaline to the bath 1 min. before an addition of choline ester inhibits the contraction to a degree depending, within limits, on the amount of catechol amine added. The method can thus be used for the quantitative determination of the adrenaline or noradrenaline content of solutions.

An aqueous solution containing compounds *X* and *Y* free from adrenaline and noradrenaline was obtained from the tumour by the octyl alcohol method described above. Part of this solution was hydrolysed with N-hydrochloric acid by the method described. The activities of the hydrolysed and the unhydrolysed solutions were then compared on the rat uterus preparation. It was found that the unhydrolysed material had an activity of only about 2% of that of the hydrolysed material. A like figure was obtained with a preparation of authentic lactyladrenaline. Blocking of the side chain hydroxyl group by ester formation thus decreases markedly the pharmacological activity of adrenaline in this test. A similar reduction in activity is obtained if the side chain hydroxyl of adrenaline is oxidized to a ketone group (Gaddum *et al.* 1949).

SUMMARY

1. Evidence is presented of the presence of lactyladrenaline and lactylnoradrenaline in addition to adrenaline and noradrenaline in an extract of an adrenal medullary tumour.

2. The esters are believed to be artifacts produced by the method of extraction employed.

3. A pharmacological test demonstrated that these esters are relatively inactive compared with the parent amines.

I wish to express my thanks to Prof. G. W. Pickering for supplying the adrenal tumour, and to Prof. J. H. Gaddum, F.R.S., for his constant interest and encouragement throughout the course of this work.

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